Development of a high throughput compatible cell assay based on the proteolytic cleavage or inhibition of the human La protein

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Abbreviations

A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
aa	Amino acid(s)
Ab	Antibody
AP	Alkaline Phosphatase
APMAA	N- (3-Amino propyl) methanylamide
APS	Ammonium per sulphate
ATP	Adenosine 5′ Triphosphate
bp	Base pair
BPB	Bromophenol blue
BSA	Bovine Serum Albumin
CBA	Cell based assay
CCD	Charge coupled device
cDNA	complementary DNA
°C	Degrees Celsius
cpm	counts per minute
CFP	Cyan Fluorescent Protein
CPP	cell permeable peptide (s)
CTL	cytotoxic T lymphocytes
Da	Dalton
DAPI	4′,-6-Diamino-2-phenylindole
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DEPC	Diethylpyrocarbonate
DIC	N,N'-diisopropylcarbodiimide
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP (s)	2′-Deoxyribonucleotide-5′-Triphosphate (s)
dsDNA	double stranded DNA
DTT	1,4-dithio-DL-threitol
ECFP	Enhanced Cyan Fluorescent Protein
ECL	Enhanced Chemiluminescence
E.coli	Escherichia coli
EDTA	Ethylendiamine tetra acetic acid
ELS	Elastase
EMSA	Electrophoretic mobility shift assay
EYFP	Enhanced Yellow Fluorescent Protein
FBS	Fetal bovine serum
Fmoc	9-Fluorenylmethyloxycarbonyl

FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GFP	Green Fluorescent Protein
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HF	Hydrofluoric acid
HIS	Histidine
HIV	Human immunodeficiency Virus
hLa	human La Protein
HOBt	N-Hydroxybenzotriazole
HRP	Horse radish peroxidase
HTS	High throughput screening
IFN	Interferon
IgG	Immunoglobulin G
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
kb	kilo base pairs
kDa	kilo Dalton
l	Litre
LB	Luria Broth Medium
M	Molar
mAb	Monoclonal Antibody
MHC	Major histocompatibility complex
MEK	Map-Erk kinase
µl	microlitre
min	minute
MTBE	Methyl tert.-butyl ether
MW	Molecular Weight
mLa	mouse La Protein
mRNA	messenger RNA
MUT	Mutant
NA	Numerical aperture
NES	Nuclear Export Signal
NiNTA	Nickel-Nitrilotriacidic acid
NLS	Nuclear Localisation Signal
NMI	N-Methyl imidazole
NMP	N-Methyl-2-pyrrolidinone
NP-40	Nonidet P-40
npg	N-propyl gallate
nt	nucleotide (s)
NTP	Nucleoside triphosphate
O.D.	Optical density
O/N	overnight
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction

PFA	Paraformaldehyde
pI	Isoelectric point
PLL	Poly L lysine
PP	Poly propylene
PTB	Polypyrimidine-tract-binding-protein
PTD	Protein transduction domains
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
Rpm	Revolutions per minute
rRNA	Ribosomal RNA
RRM	RNA recognition motif
RT	Room Temperature
sec	Second (s)
S. cerevisiae	Saccharomyces cerevisiae
SDS	Sodium dodecyl sulphate
SPPS	Solid phase peptide synthesis
ssDNA	Single stranded DNA
Taq	Thermus aquaticus
TBS	Tris-buffered saline
TBST	TBS-Tween 20
TEMED	N,N,N`,N`-Tetramethyl-Ethylendiamine
TIBS	Triisobutylsilane
TNF	Tumour necrosis factor
TFA	Trifluoroacetic acid
Tris	Tris- (Hydroxymethyl)-Aminoethane
tRNA	transfer RNA
Tween 20	Polyoxyethylene Sorbitan Monolaurate
U	Unit (s)
UV	Ultraviolet
v/v	volume per (total) volume
v/w	volume per (total) weight
VEGF	Vascular endothelial growth factor
WB	Western Blot
WT	Wildtype
w/v	weight per volume
w/w	weight per weight
YFP	Yellow fluorescent protein

DNA codes used

Single letter code	DNA base
A	Adenosine
C	Cytidine
G	Guanosine
T	Thymidine

Amino acid codes used

Single letter code	Three letter code	Amino Acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

1 Introduction

1.1 Chemical Genetics as a method to study biological systems

Genetics has been used widely to study biology by manipulating the biological system at the level of the gene. It has been the benchmark enabling us to understand how genes and proteins function. In traditional genetics or **'forward genetics'**, the genome of a model organism is randomly mutated. Mutants that produce a change in a desirable phenotype or trait, such as growth, appearance or behaviour, are used to discover the identity of genes responsible for producing the phenotype. Advances in molecular biology have led to a second type of genetic approach called **'reverse genetics'** where an already identified gene is mutated or deleted and the resultant phenotype studied, thereby providing a picture of the role of that gene in the organism.

Traditional genetics also has some limitations. One major obstacle of traditional genetics is that mutations are usually constitutive. Mutations in essential proteins often lead to lethality at early stages of life and make it impossible for subsequent studies. In the case of non-lethal mutations, mutant organisms are often able to compensate for the loss of the gene, which obscures the effect of the original mutations. In mammals, genetic approaches are limited by their large diploid genome, large physical size and slow rate of reproduction. Another limitation of the genetic approach is that most mutations are not conditional — they cannot be turned on or off at will. Conditional mutations such as temperature-sensitive mutations usually cause an environmental stress to the organism, such as a temperature shift, which itself can have marked consequences and may mislead the interpretation of results. In addition, organisms that carry constitutive mutations may have time to compensate for their effects by upregulating related genes, which can obscure the initial effects of a mutation.

In recent years, **Chemical Biology** has opened new avenues to overcome the limitations posed by classical genetics approaches. It is defined as the functional and mechanistical investigation of biological processes using chemical compounds as modifiers of biological activity. **Chemical Genetics** is the discipline that aims at studying gene function at the level of their products, the proteins, by identifying chemical compounds which induce or revert biological phenotypes. **Chemical genomics/Chemogenomics** is thus defined as the

systematic identification of small molecules that interact via a specific molecular recognition mode with target proteins encoded by the genome. The term chemogenomics is applied more specifically to target gene families in drug discovery.

Chemical genetics complements the existing classical genetic strategies (e.g. mutagenesis) allowing for fine tunable *in vivo*-modulations of protein functions and cellular processes. It involves the study of biological systems using small molecule ('chemical') intervention, instead of only genetic intervention. **Small molecules** are used to induce alterations in gene products in mammalian systems, in a manner similar to mutations. This approach involves screening with chemical probes that potentially could interact with any target in the genome, while trying to identify specific phenotypes. On the other hand, chemical genetics is also analogous to introducing specific gene disruptions, here compounds that are known to specifically interact with a given target are used in broad phenotype screens to help identify the physiological role of that target.

Chemical genomics has several outstanding advantages over classical genetics and molecular techniques for studying gene functions. Standard genetics approaches target one gene at a time and provide limited opportunity to control the extent of the downstream cellular effects. By contrast, chemicals can be targeted with spatio-temporal precision against a selected spectrum of proteins. They can be applied in defined dosages to distinct cells, organs, or developmental stages, often with rapid response times and reversible effects. Since the mechanism of action of chemical switches is similar across a range of organisms, their identification is of great interest for researchers working with different model systems. Finally, the chemicals can be used to inactivate a family of proteins with related sequences or structures in a single step. These ''chemical family knock-downs'' are being increasingly used as the method of choice for the functional characterisation of paralogous genes with redundant functions (1; 2).

Such a chemical genetics study requires the involvement of at least three things: a selective small molecule, its protein partner, and biological screening (3). Instead of starting with a gene, the chemical genetics approach starts with small drug-like molecules to screen for protein targets and study their role in biological processes in general, as well as in disease related pathological situations. The result is a validated gene function and a potential drug candidate all in one step.

Small molecule compounds that specifically inhibit individual targets can be used to speed up this unravelling of the molecular dissection of the biology underlying various disease states, thereby providing multiple opportunities for "genome or proteome mining". Cell permeable and selective small molecules can be used to perturb protein function rapidly, reversibly and conditionally with temporal and quantitative control in any biological system. The screening of small molecules against specific targets can thus aid in determining more potent drug leads. This chapter deals first with understanding the different types of approaches used in classical and chemical genetics which in turn determine the type of screening approach that would be used. This knowledge is essential in order to facilitate the synthesis of the respective small molecule libraries to be screened. This chapter will also deal with the presentation of small molecules in array formats and the development of assays to screen for active biological targets. Particular attention has been paid to illustrate the significance of each section through examples cited through literature surveys. This study involves techniques from cell biology, molecular biology, biochemistry and chemistry thereby illustrating the interdisciplinary nature of this work.

1.1.1 Classical and chemical genetics approaches

The two approaches in traditional genetics have been introduced in the previous section. **Forward genetics** is characterised by random mutations followed by phenotypic screening and gene identification (as seen in figure 1-1). In **reverse genetics**, deletion or mutation is done of a specific gene followed by phenotypic characterisation of this mutation. As observed in traditional genetics, the early stage of chemical genetics primarily involved '**forward chemical genetics'**, in which natural products or a collection of synthetic compounds are screened for desired phenotypes, such as inhibition of tumour growth *in vitro.* The outcome of these studies pointed out the high specificity and potency of natural products towards important cellular proteins, leading the way to identify drug targets using chemical compounds as probes.

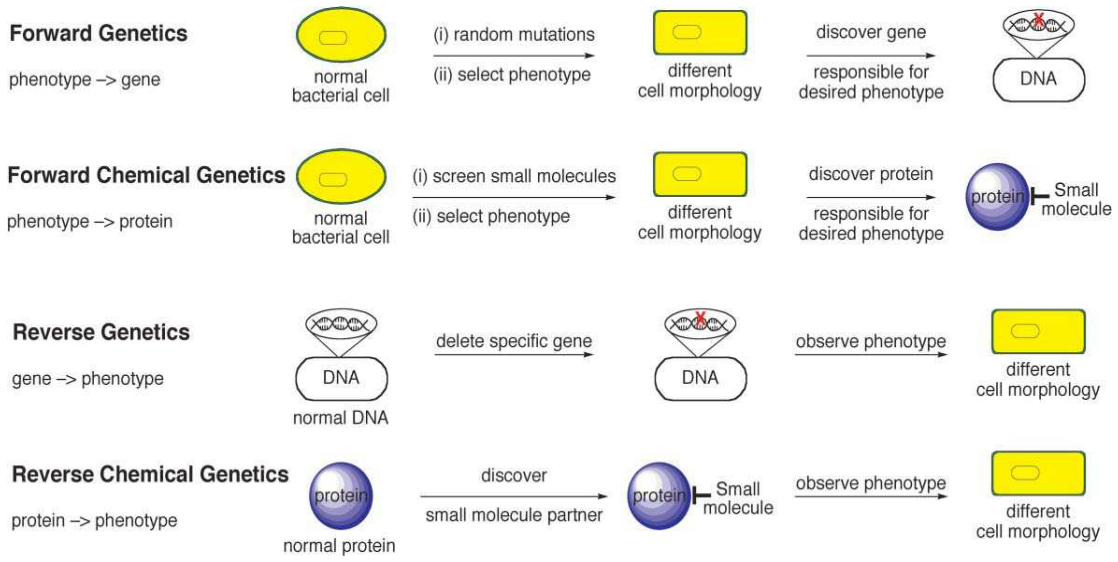

Figure 1-1. Differences in the classical and chemical genetics approaches
(adapted from (3)).
Forward genetics and forward chemical genetics employ the use of either random genetic mutations or screening of small molecule libraries to discover the respective gene or protein responsible for the desired phenotype. Hence, in the forward approach the direction is from phenotype to the identification of the molecular component. In the reverse approach, the phenotype resulting from the mutation or deletion of an already identified gene or inhibition of a protein by its known small molecule interaction partner is studied.

Thus, in **forward chemical genetics**, small molecules are screened for the desired phenotypic effect on the biological system under investigation, thereby identifying the proteins and genes responsible for the phenotype being investigated. On the other hand, the **reverse chemical genetics** employs the use of small molecules against a protein (gene product) of interest in order to identify any resultant phenotypes. Classical genetics approaches, never the less, are still used today such as in understanding circadian rhythms in the fruit fly, Drosophila, using the forward genetics approach (4; 5) and the characterisation of the Myosin V gene in Drosophila (6) using reverse genetics.

1.1.2 Application of small molecules in chemical genetics approaches

In chemical genetics, with the advent of newer, more specific chemical reagents, it has been suggested that these compounds be used as baits to determine the interacting proteins and their coding genes responsible that are thought to be responsible for the observed effect (7). A medicinal drug compound having a molecular weight of less than 1000 Daltons, and typically between 300 and 700 Daltons is considered a **small molecule** (8).

Genetic analysis of the yeast, *Saccharomyces cerevisiae* has contributed much to our knowledge of eukaryotic cell regulation. By using phenotypic readouts such as cell viability and morphological alterations, the genetic interaction between two genes can be examined by mutations in both genes, which has been a powerful tool in yeast to discover and characterise biological pathways. (9; 10) Conditional loss of function of proteins by chemical inhibitors in yeasts has greatly facilitated such genetic analyses (11; 12). The examples listed in the table 1-1 below depict how chemical genetics has furthered our understanding of biology as we know it today through the use of small molecules.

Drug	**Function known through classical studies**	**Use of Chemical genetics has revealed**	**References**
Cyclopamine	Teratogen and an inhibitor of the Sonic hedgehog (Shh) pathway	The role of cyclopamine in the Shh pathway through Smoothened was unravelled. Further elucidation of the role of the Shh pathway in hair follicle, limb and gastric gland development has been made possible.	(13-16)
Lactacystin	Protease Inhibitor with 20S proteasome as the sole target	Discovery of the proteasome in pathways such as antigen presentation, cell cycle control and cell fate determination	(17; 18; 19)
Leptomycin B	Antifungal and anti-bacterial activity	Leptomycin B inhibits CRM1, a protein required for nuclear export	(20; 21)
Rapamycin	Immunosuppressant with TOR (target of rapamycin) proteins as its targets	Identification of TOR dependent cellular factors such as S6 kinases and Gln3	(22; 23)
Tacrolimus or FK 506	Immunosupressant	FK 506 inhibits T lymphocyte signal transduction and IL-2 transcription by inhibiting the protein phosphatase Calcineurin	(24; 25)

Table 1-1. Chemical genetics has helped further the knowledge gained by classical genetics studies

1.1.3 Phenotype based forward chemical genetics

Small molecules can help unravel key targets in biological pathways and this can be truly appreciated by the following examples. **Anti-mitotic agents** are important in anti-tumour studies. The majority of the known inhibitors of mitosis act on tubulin during mitotic spindle formation (26). In order to identify small molecules that can interact with other proteins important for mitosis, Mayer et al. (28) developed a screening procedure that targeted nucleolin phosphorylation in cells entering mitosis. Of the 139 positive compounds they obtained from this screen, they were able to narrow down one compound, Monastral. It arrested cells in mitosis with a monopolar spindle by inhibiting the mitotic motor protein kinesin Eg5, which is required for spindle bipolarity (27; 28). Thus a **phenotype based forward chemical genetics** screen helped identify a novel antimitotic molecule. Following this study, other selected inhibitors of Eg5 which have been reported are: dihydropyrazoles (29), terpendole E (30), and dimethylenastron (31). Similarly, screening of a microbial products library for shortened telomere length in yeast yielded three structurally unrelated antibiotics, chrolactomycin, UCS1025A, and radicicol (32). Of these, chrolactomycin was also found to inhibit human telomerase. Thus phenotype based forward chemical genetics approaches could be used in a yeast-based assay to discover small molecules acting on human telomerase.

1.1.4 Target based reverse chemical genetics

Protein kinases represent one of the largest protein families in the human genome with current estimates (excluding mutants and splice variants) running in excess of 500 members. Members of the protein kinase family are crucial elements in cellular signalling pathways, and are therefore of interest as drug targets for cancer, inflammation, etc. These enzymes play a pivotal role in gene expression regulation, cellular proliferation and cell differentiation. Not surprisingly, dysfunctions of protein kinases are associated with numerous severe pathological states and specially designed small molecule inhibitors have several potential therapeutic applications, notably in the areas of diabetes, immune diseases and cancer. Many kinase inhibitors have been discovered from natural products or through medicinal chemistry programs. Examples of small molecule kinase inhibitors used to probe cellular signalling pathways are as listed below (table 1-2).

Small Molecule Inhibitor	Target	Reference
Flavopiridol	cyclin dependent kinase	(33)
Gleevec/ Imatinib	Bcr-Abl/ c-Kit	(34; 35)
U-0126 or PD98059	Mitogen Activated Protein Kinase Kinase	(36)
Wortmannin	PI3 kinase	(37)
Y-27632	Rho associated kinases	(38)

Table 1-2. Kinase inhibitors and their targets

Similarly, protein phosphatases are key regulatory enzymes that dephosphorylate specific amino acid residues and are critical regulators of signal transduction under normal and pathological conditions such as cell growth and differentiation, cell cycle, metabolism, immune response and cytoskeletal function. Natural products such as dephostatin (from Streptomyces species) and karanjin (*Pongamia pinnata* fruits) are protein tyrosine phosphatase inhibitors that are being looked into for further modifications to improve their suitability as drugs (39).

Target based reverse chemical genetics too has distinct advantages over the classical reverse genetics approach. MEK kinases comprise a family of related serine–threonine protein kinases that regulate mitogen-activated protein kinase signalling pathways. These stress-activated kinases have been implicated in apoptosis, oncogenic transformation and inflammatory responses in various cell types. To define the function of MEK1 in cell cycle progression, cell growth and cell morphology, the effect of PD 184352 on these processes was tested in colon tumour cells. These studies found that MEK1 activity is required for: the progression of cells from the G1 to S phase of the cell cycle, anchorage independent growth, cell scattering and the conversion of cells from a flattened to a round morphology. Treating colon tumour-bearing mice with PD 184352 caused tumour size to shrink, indicating that MEK1 function is also required for *in vivo* colon tumour cell growth (PD 184352 had no effect in mice with leukaemia). In this study, a small-molecule MEK1 inhibitor was discovered and used to define the function of MEK1 in tumour processes. It is interesting to note that in the analogous reverse genetic approach, Mek1-deficient mouse embryos died in early embryogenesis and because it is an essential gene, its function could not be studied in

tumour processes from adult mice by reverse-genetic methods. In such cases, reverse chemical-genetic methods complement and extend reverse-genetics methods for studying specific gene product functions *in vivo*.

Genome-based unbiased chemical screening will help discover small molecules that target novel proteins in specific pathways and networks. **Molecular target identification** is one of the key elements of chemical genomics screen with small molecules. To identify such target proteins, several approaches have been developed such as high-density, proteome-based affinity purification, genetic/genomic screening and *in vivo* and *in vitro* expression screening. It is necessary to systematically analyze these various approaches to test their feasibility for identification of the proteins whose activity is affected by chemicals.

1.2 Sources and synthesis of small molecules

1.2.1 Natural compound libraries

There are a number of potential sources of small molecule collections. Traditionally, nature has been a rich source of molecules that effect biological systems, many of which act on specific protein targets. Natural products, which are both complex and diverse in structure, have been used for centuries as medicines and have had a profound impact on human lives. The introduction of the sulphonamide antibiotics in the 1930s and penicillin in the 1940s revolutionised medicinal practice by dramatically decreasing the fatality rates associated with bacterial infections. This thus led to the search for new natural products with antibacterial properties. Natural products also are likely to have evolved to penetrate cell membranes and interact with specific protein targets and their bioactivity is their advantage. Natural products screened for today also have antifungal and antiviral properties and are also useful in the oncological, cardiovascular, neurological and immune related diseases (40). The specificity of antibodies, T cell receptors as well as hormone and steroid receptors is also known to be flexible with respect to the structures they bind. Antigen presenting MHC molecules as well as proteasomes and peptide transporters are known to bind to peptide libraries from self proteins. Nature thus evolved the concept of diverse ligand-receptor based libraries exploited in order to identify lead drug targets today.

Novel natural products from **microbial** sources offer lead structures of the highest molecular diversity and complexity (41). Microbial peptide antibiotics are usually isolated

from their metabolites as mixtures of closely related molecules with some micro heterogeneity. Natural products produced by **plants** also offer a large variety of diversity within the same class of organic compound found in nature (42). Structurally related compounds have the ability to perform similar biological functions and are thus available as differently substituted parent compounds. Several **marine**-derived compounds such as cemadotin (microtubule interfering peptide, from sea slug) and yondelis (DNA interacting isoquinolone, from sea squirt) are in clinical trials, particularly as anti-cancer agents (43-46). Despite their diversity, natural products are obtained in low quantities. Also, difficulties associated with purification and characterisation, as well as their structural complexity makes natural product chemical derivatisation, a process especially relevant to drug discovery, extremely challenging. Thus slowly the focus shifted to generating synthetic analogue compound libraries which were natural product derived but had improved properties.

1.2.2 Strategies for the synthesis of libraries

Modern drug discovery often involves screening small molecules for their ability to bind to a preselected protein target. In this strategy, sets of discrete compounds are prepared simultaneously in arrays of physically separate reaction vessels or micro-compartments without interchange of intermediates during the assembly process. Several libraries are based on or inspired by the structures of natural compounds and are synthesised in an attempt to further develop more potent and active drug leads (47). For example, new classes of anti- tuberculosis compounds currently in preclinical testing include derivatives of the natural products capuramycin, oxazolidinones and beta-sulfonylcarboxamides (48).

1.2.2.1 Target oriented synthesis (TOS) and focussed library synthesis

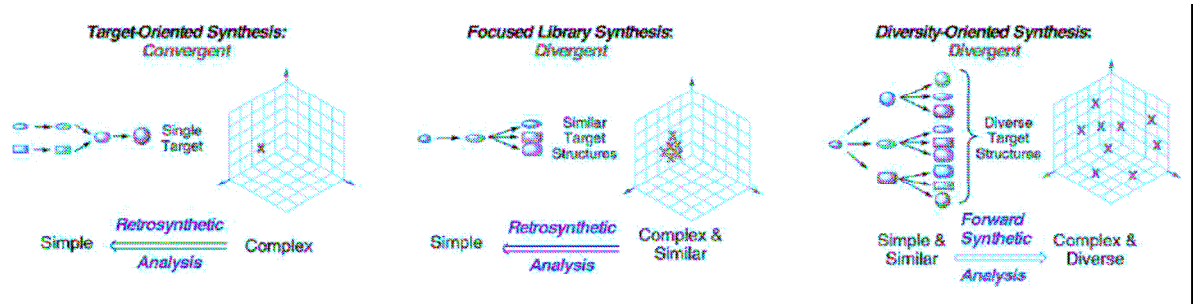

Figure 1-2. Comparison of target-oriented synthesis (TOS) versus focussed library synthesis and diversity-oriented synthesis (DOS) (adapted from (49))
In figure 1-2, the three approaches to synthesizing small molecules have been depicted. This includes the convergent TOS approach as well as the divergent focussed library synthesis and DOS approaches. The colours or shapes imply no specific meaning except that each unit represents a different compound. The grid represents the 3 dimensional chemical space. TOS and focussed library synthesis approaches transform a more complex target molecule into simpler structures using retrosynthetic analysis. In DOS, forward synthetic analysis is used to transform simpler starting materials into a more complex collection of compounds.

Target oriented synthesis aims to synthesise a single target molecule, whereas a focussed library generates structurally similar target structures. Retrosynthetic analysis concepts focus on the existence of a defined target structure. In figure 1-2, the first approach uses target-oriented synthesis (TOS) and relies primarily on nature to discover small molecules with useful, macromolecule-perturbing properties. Natural compounds can be identified in screens of extract mixtures, isolated, and then structurally characterised by using a variety of spectroscopic techniques. Once such a structure has been identified, it can become a target for chemical synthesis. TOS aims to access a precise region of chemical space, which is often defined by a complex natural product known to have a useful function. Target-oriented syntheses of small molecules, individually or as collections (focussed libraries), can be planned effectively with retrosynthetic analysis. **Retrosynthetic analysis** technique involves transforming the structure of a synthetic target molecule to a sequence of progressively simpler structures along a pathway which ultimately leads to simple or commercially available starting materials for a chemical synthesis.

With **focussed screening**, it should also be possible to use an assay that is more appropriate, rather than one that works well at a large scale. For example, it has been common for *in vitro* isolated receptor-binding assay formats to be developed for high-throughput screens, even

when greater value could be derived from a whole-cell functional approach. The second approach uses either medicinal chemistry or combinatorial chemistry and aims to explore a dense region of chemical space in proximity to a precise region known to have useful properties. The source of the starting or lead compounds can vary and may include a natural product, a known drug, or a rationally designed structure developed from a mechanistic hypothesis and/or a crystal structure of a macromolecule of interest. In this thesis, both target oriented synthesis and focussed library synthesis approaches will be used to screen for phenotypes and peptides.

1.2.2.2 Diversity oriented synthesis (DOS)

It is critical to the chemical genetics approach to have a library of compounds that have a high probability of being relatively selective; otherwise the ability to interpret the results becomes at least as complex as deciphering highly polygenetic phenotypes. To address this, diversity-oriented synthesis (DOS) has been proposed to provide arrays of complex small molecules that are easily synthesised. Drug discovery can also involve screening small molecules for their ability to modulate a biological pathway in cells or organisms, without regard for any particular protein target. This process is likely to benefit in the future from an evolving forward analysis of synthetic pathways, used in diversity oriented synthesis, that leads to structurally complex and diverse small molecules. **Forward synthetic analysis** is a problem-solving technique for transforming a collection of simple and similar starting materials into a collection of more complex and diverse products.

DOS concerns the efficient synthesis of structurally diverse (and complex) small molecules (i.e. where the molecules differ in their (i) attached groups, (ii) stereochemistry, (iii) functional groups and (iv) molecular frameworks). Synthetic pathways in DOS are branched and divergent and the planning strategy extends simple and similar compounds to more complex and diverse compounds. In DOS there is no single target structure and therefore retrosynthetic analysis cannot be used directly and a forward synthetic analysis algorithm is required.

Structural complexity is important because many of the small molecules known to disrupt protein-protein interactions are structurally complex natural products. The Shair lab recently demonstrated the power of diversity-oriented synthesis combined with phenotypic screening in the synthesis of a library of compounds based upon the natural product

galanthamine (50). From this library, they discovered a compound, **secramine**, which blocked protein trafficking from the golgi apparatus to the plasma membrane. Another significant result from this work was the lack of inhibition of protein trafficking by the parent compound galanthamine at concentrations as high as 100 mM. Diversity-oriented synthesis in combination with phenotype-based assays has emerged as a powerful tool for the study of biological systems.

1.2.3 Synthesis of small molecule compound libraries using combinatorial chemistry methods

Structural diversity is essential for lead generation, as compounds that look the same structurally are likely to share similar physical and biological properties. Early efforts to generate chemical diversity focussed on chemically synthesised peptides because they are composed of the same building blocks as proteins and were therefore thought to be good candidates for ligands that bind to proteins. Using combinatorial chemistry, a large number of variants of one particular core structure were created by combining different building blocks with suitable chemical reactions. **Combinatorial chemical synthesis** is thus a process by which multiple compounds (chemical libraries) are generated simultaneously, in a predictable fashion, by using techniques that involve parallel chemical transformations. However, the number of compounds that could be individually synthesised by conventional chemistry was limited by this approach.

1.2.3.1 Solid phase synthesis

In the 1960's, Merrifield introduced the concept of **solid phase synthesis** (51; 52). Synthesis on solid supports essentially consisted of three key elements: the solid support, the linker element and the compound attached to the linker. In general, synthesis on solid supports thus involves two additional steps in synthesis compared to synthesis in solution involving the attachment of the starting material to the linker at the start of synthesis and the cleavage of the finished product from the linker at the end of synthesis. However, the types of chemistry performed in order to synthesise these libraries is governed by the types of linkers used and their stability during reactions. Libraries containing up to 10,000 and even more

compounds may use the spatially separate synthesis approach where compounds are synthesised in parallel but in separate reaction vessels such as microtiter plates. Biological evaluation of such libraries can provide specific information about each compound in the library.

In solid phase synthesis, a pin or bead technique permits the synthesis of different molecules on each pin (i.e. "one molecule-one bead" (53)). The scale of the different peptides and oligonucleotides synthesised by the repetitive coupling of suitable individual monomeric building blocks was now sufficient for analysis (54). Alternatively, libraries containing more than 100,000 compounds may also be synthesised using photolithographic methods that allow for light directed spatially addressable parallel chemical synthesis (55). In this method, a silica wafer serves as a solid support. In general, larger libraries of compounds are synthesised by employing pooling strategies (56). This strategy uses beads and a repetitive splitting and pooling steps in order to generate all possible variations of different sequences of building blocks. Another pooling strategy developed by Houghten deconvolutes a soluble library after cleavage from the solid support (57). This method has successfully been used to identify high affinity ligands. However, since biological activity depends on the abundance of active compounds in each pool, the pool that shows greatest biological activity may not be the one with the most potent compounds.

1.2.3.1.1 Solid-phase peptide synthesis (SPPS) using the tBOC method

There are two main protocols that have been used for the chemical **solid-phase synthesis of peptides (SPPS)**. When R. B. Merrifield invented SPPS in 1963, it was according to the **tBoc method**. T-Boc (or Boc) stands for tert-butyloxycarbonyl. To remove Boc from a growing peptide chain, acidic conditions are used (usually neat TFA). Removal of side-chain protecting groups and the peptide from the resin at the end of the synthesis is achieved by incubating in hydrofluoric acid (which can be dangerous); for this reason Boc chemistry is disfavoured nowadays. However for complex syntheses Boc is favourable. When synthesizing non-natural peptide analogues which are base-sensitive (such as depsi-peptides), Boc is still the method of choice.

In SPPS the monitoring of the completion of the Boc cleavage and of the coupling reaction is advantageous. Reliable methods detecting minute amounts of unreacted amino groups are essential for the monitoring of the coupling reaction. A range of colour tests for the

qualitative monitoring of the coupling reaction has been developed. The Kaiser test, which is based on the reaction of ninhydrin with amines and which is a very sensitive test for primary amines, is very often in use. Finally, the peptide linker-support is cleaved to obtain the peptide. Ideally, the cleavage reagent should also remove the amino acid side-chain-protecting groups. In the most commonly employed anchoring system to peptide acids and amides are the 4-hydroxymethylphenylacetic acid (PAM) linker and 4-methylbenzhydrylamine (MBHA) resin. The peptide-resin anchorage and side–chain protecting groups are cleaved by the use of anhydrous HF. Linkers which are cleaved under non-acidolytic conditions are also known. Among these is hydroxyl-crotonyl-aminomethyl (HYCRAM) where for the cleavage tetrakis-(triphenylphosphine)-palladium ($Pd(PPh_3)_4$) is used.

1.2.3.1.2 Solid-phase peptide synthesis (SPPS) using the Fmoc method

The second protocol uses the 9-fluorenylmethyloxycarbonyl (Fmoc) group for N-amino protection. The **Fmoc method** was introduced by R. C. Sheppard in 1971. Fmoc stands for 9-fluorenylmethoxycarbonyl which describes the Fmoc protecting group. To remove the Fmoc-group from a growing peptide chain, basic conditions (usually 20% piperidine in DMF) are used. Removal of side-chain protecting groups and peptide from the resin is achieved by incubating in trifluoroacetic acid (TFA). The main advantage of Fmoc chemistry is that no hydrofluoric acid is needed. It is therefore used for most routine synthesis.

During the synthesis, Fmoc is split off by a short treatment with piperidine in DMF. For coupling reactions DCC or DIC can be used and for the qualitative monitoring of the coupling reaction Kaiser test can be used. Side-chain protections that are compatible with N-protection are removed at the same time as the appropriate anchoring linkages typically by the use of TFA. The Fmoc protocol is especially recommended for the synthesis of acid sensitive peptides and derivatives. The choice of an adequate combination of protecting groups/solid support is the first step to achieve a successful synthesis.

The products of solid phase synthesis can be cleaved from the backbone matrix for solution screening (which is essential when the screening target is a cell), or the most active molecules displayed on the polymer surface may be detected using **molecular targets** (receptor, enzyme, antibody) pre-tagged with a means of detection (visible colour, fluorescence, radioactivity, chromophore, etc.) and then isolated and identified. Synthesis on

solid supports reduces time consuming purification and isolation steps as compared to the individual synthesis of compounds in solution.

A **random library** may be defined as a library prepared from building blocks without bias towards a particular target. These are of a generic nature and are applicable to any screening tasks. Random peptide libraries can be displayed using the technique of phage display in order to facilitate screening of a phenotype. Peptide libraries have yielded ligands for many different protein targets such cyclin dependent kinases (58), Calmodulin (59) and fibroblast growth factors (60). Over the past decade, methods for the synthesis of chemical libraries of non-peptidic, low-molecular weight organic compounds have also been developed (61). **Organic compounds libraries** have the advantage of often being able to permeate the plasma membrane of target cells. These libraries have yielded compounds that are active in cell based assays (62). Mueller et al. have taken this a step further and selected gene therapy vectors using random peptide libraries displayed on adeno associated virus (63; 64). More **dedicated peptide libraries** are biased for particular domains and are constructed for more detailed preferences of ligand analysis.

1.2.4 Combinatorial biology methods

Although combinatorial biology methods will not be used in this thesis, combinatorial libraries can also be generated based on these methods. Combinatorial biology depends on the ability to link polypeptides to their encoding DNA. The methods for generating DNA-encoded polypeptide libraries can be divided into two groups. *In vitro* methods use libraries in which polypeptides are accessible to exogenous ligands. Combinatorial biology exploits the ability to use libraries for in direct *in vitro* binding selections with immobilised target ligands and also enrich for polypeptides with particular binding specificities and affinities. **Phage display** is the predominant method for *in vitro* combinatorial biology. The method stems from the observation that polypeptides fused to certain bacteriophage coat proteins are displayed on the surfaces of phage particles that also contain the cognate DNA. Libraries of 'fusion phage' can be rapidly sorted to obtain clones with desired binding properties and phage can be readily amplified by passage through a bacterial host (65). Another approach to DNA-encoded libraries is provided by **cell surface display** in which polypeptides are displayed directly on bacterial or yeast cells that also contain the encoding plasmid DNA (66).

Phage-display combinatorial peptide libraries are typically screened by affinity selection with a particular target protein. In many cases, it is possible to identify, from the affinity-selected peptides, members with a sequence that closely resembles segments (epitopes) of a natural interacting partner of the protein. A practical consequence of this phenomenon, termed 'convergent evolution', is that one can search whole genome databases for proteins containing segments that match consensus sequences shared by the selected peptides, and then experimentally determine whether or not they interact with the target. As an example, combinatorial peptide libraries have proven useful in defining the optimal ligand preferences of protein interaction modules, such as various SH2, SH3 and WW domains within proteins of interest, and suggesting potential interacting proteins (67; 68).

In contrast, *in vivo* methods use polypeptide libraries that are expressed inside living cells along with a target protein of interest. An interaction between a particular library member and the target protein is detected by virtue of an effect on the host cell, such as a selective growth advantage, or changes to a physical property of the host cell. *In vivo* methods can be defined as those in which the protein-protein interaction under study is detected inside a living cell. The most widely used *in vivo* method for the detection of protein–protein interactions is the **yeast two hybrid system** (69). Interaction between the two proteins of interest (the 'target' and the 'bait') results in functional assembly of the two components of a signalling system: a DNA-binding domain and a transcriptional activation domain. As a result, transcriptional activation of a reporter gene occurs leading to a detectable phenotype, which can be screened for or directly selected. Although this describes the 'standard' yeast two-hybrid assay, it has given rise to a several variations (70; 71).

1.3 Presenting small molecule libraries as arrays

Macroarrays, the immediate predecessors to microarrays, are used for the expression analysis of tens or hundreds of genes (72). Macroarrays are usually printed on nylon membranes approximately 8 x 12 cm in size containing up to 5000 spots, each greater than 300 microns in diameter. Microarrays, on the other hand, usually have 10,000 - 40,000 spots that are less than 200 microns in diameter in a 1.5 cm^2 area. A recent popular approach for associating genes with specific signalling pathways is the use of **nucleic acid microarrays**, which contain immobilised cDNA fragments or short oligonucleotide probes. Microarrays

can comprise the entire human genome and novel techniques enable the detection of a specific target within several thousand transcripts (73). Using these tools, scientists are now in a position to identify genes that are specifically up- or downregulated in response to a defined stimulus (74).

A review by Chiosis and Brodsky outlines some of the different methods used to present **small molecules in a microarray format** (75). Some of the key points to take into consideration while printing are compatibility with pre-existing library formats (mostly compounds are dissolved in DMSO) and prevention of cross-contamination by printing small molecules on glycerol droplets and aerosol deposition (76). Printing chemical libraries on microarrays is a well-known process. However, DNA chips and microarrays for genotyping and expression profiling give no information about the activities of enzymes that can be regulated by post-translational modifications or cleavage state. Microarrays have also successfully been used to find differentially expressed genes by analyzing large sets of patient samples, or by comparing normal with diseased tissues (77; 78).

Recently, **protein arrays** have also been developed that have the potential to detect post-translational changes in proteins, such as phosphorylation, acetylation, glycosylation, ubiquitination or other covalent protein modifications (79; 80). These modifications, which do not require *de novo* transcription, would be missed by analyzing changes exclusively at the mRNA level. Protein microarrays have used the capture of proteins to libraries of immobilised DNA sequences, peptides, antibodies, chemical motifs, or other proteins (79; 81). The three major formats for protein arrays currently use plain **glass slides** (79), **3D gel pad** chips (82) (''matrix'' chips), or **nanowell** chips (80; 83).

Other types of arrays, such as **sensor arrays**, directly translate molecular interactions into optical or electrical signals (84). **Aptamers**, which are small nucleic acid molecules, and **antibodies** can function as biosensors for numerous diagnostic and screening applications (85). Both capture tools can recognise targets ranging from small molecules to complex multimeric structures with high specificity *in vitro* and *in vivo* (86). A systematic screen for potent aptamer-DNA sequences yielded, for example, high affinity biosensors for anthrax spores (87). Although all of the tools described earlier are powerful tools to associate genes and their products with certain pathways or processes, in most cases this association remains correlative. Further studies will be required to determine whether the role of the gene of interest is also causal in, for instance, the progression of a disease. Non-DNA

microarrays, such as protein, peptide and small molecule microarrays, pose some fundamental obstacles that limit their rapid and widespread implementation as an alternative bioanalytical approach. These include for example, the prerequisite for numerous proteins in active and purified form, ineffectual immobilization strategies and inadequate means for quality control of the considerable numbers of multiple reagents. Below are lists of the different types of arrays and the different formats (supports) and the methods used to generate them.

Array type	**Uses**	**Reference**
Antibody arrays	Cytokine profiling, identification of markers in cancer	(88; 89)
Protein chips	Detection of cancer and protein- protein interactions	(90; 91)
Peptide arrays	Identification of epitopes, study of protein - protein interactions	(92-94; 95)
Peptide Nucleic Acid arrays	Detection of mismatch mutations and genetically modified organisms	(96; 97)
Lipid arrays	Detect mediators of brain inflammation	(98)
Oligosaccharide/ Carbohydrate arrays	Detection of cancer associated carbohydrates and antibodies against diseases such as arthritis	(99; 100)
Chemical microarrays	Screen inhibitors of enzymes such as elastase	(101)
Sensor arrays	Immunoassay of disease markers such as in cancer	(102)
Array support	**Uses**	**Reference**
Plain glass slides	Printing chemical microarrays	(76)
Filters	Peptide arrays	(103)
Microtiter plates	Peptide arrays	(104)
HydroGel chip	Used in DNA microarrays	(105)
Nanowell chips	DNA microarrays	(106)
Compact disc based microarrays	Peptide nucleic acid arrays	(107)
Method used to generate arrays	**Uses**	**Reference**
Pin printing	G protein coupled receptor (GPCR) arrays	(108)
Photolithographic synthesis	Generation of peptide arrays	(109)
Covalent immobilisation	Immobilisation on microtiter plates in protein and sensor arrays	(110)
Self assembling libraries	Protein and chemical microarrays	(111; 112)
In situ synthesis	Generation of small molecule microarrays	(113)

Table 1-3. Different types of arrays and the supports and methods used to generate them.

1.4 Assay Platforms

With the different genetic screening and array approaches being discussed in the previous sections, the focus of this section will be on the different assay methods that facilitate these screening approaches and further their application to biological systems. Several key factors contribute to the successful use of small molecules to explore biology. The relative selectivity of chemical probes that will be used must be known, the correlations between the cellular readouts used and the pathway or phenotype that is being assessed must be independently validated, all data must be fully integrated, allowing the user to navigate through biological pathways and supporting literature, assay results and detailed information on compounds. An assay can thus be defined as a method that is used to translate a biological effect into a measureable physico-chemical signal. One of the first steps in using or developing an assay is choosing between a "cell free" (biochemical) assay or a "cell based assay" system.

1.4.1 Cell free assays

In biological assays, the challenge is to be able to conduct the assay while maintaining the structural integrity of the biological molecule being studied. Several cell free assays use egg extracts to study intricate protein interactions. Egg extracts are naturally cell cycle synchronised and mimic the complex interplay of proteins that support cellular DNA replication and naturally-regulated DNA damage checkpoint activation (114). On the other hand, study of biological processes such as receptor mediated endocytosis mediated by cell surface receptor-ligand interactions requires the use of broken cell systems (115). Cell free assays provide advantages such as eliminating the need to separate bound and free forms of ligands (116). Table 1-4 lists some examples of cell free *in vitro* assays.

Cell free *in vitro* assays	Example	Reference
Assays based on direct interaction		
Scintillation proximity assays (SPA)	Lysosome to phagocyte targeting, identification of inhibitors of CD28 necessary for T-cell response	(117; 118)
Electro-chemiluminescence	Integrin-ligand interactions where ruthenium-conjugated monoclonal antibodies are used for the detection of either purified integrins or, more conveniently, integrin-expressing cell lysates, which are captured on beads coated with extracellular matrix or vascular ligand proteins	(119)
Absorbance assays based on enzyme activity		
Enzyme linked immunosorbent assay, ELISA in solution	Antibody screen against human interferon alpha, screen molecules that compete with the natural ligands for binding to the active site of the Type-I interleukin-1 receptor	(120; 121)
ELISA on solid phase	Development of bioassays for bovine herpes virus and hepatitis B diagnosis	(122; 123)
Reporter based assays		
Luminescence based assays	Measure toxin activity by inhibition of protein synthesis using rabbit reticulocyte lysate containing luciferase mRNA	(124)
Fluorescence based assays	Fluorescence polarization based molecular binding assay using the chemokine receptor CXCR4 and integral molecular lipoparticles	(125)
Scalability of cell free assays		
High throughput assays	High-throughput AHL screening of bacteria or metagenomic libraries using cell free beta galactosidase expression, cell free assay for rapid oligo screening for the identification of new drug targets in pathogenic microbes	(126; 127)
Miniaturised assays	High-density peptide-aptamer microarrays to detect proteins electrically in whole cell lysates	(128)

Table 1-4. Examples of cell free *in vitro* assays

The Springer handbook of Nanotechnology (129) enumerates how G protein coupled receptors can be used as a biosensor in cell free assays. The miniaturization of cell free assays is only limited by the sensitivity of the reporter being assayed for. Cell free

fluorescence based assays are discussed in more detail in section 1.5, table 1-7. Virtual screening can also be used prior to testing in cell based assays to identify or narrow down targets from a known library of compounds. It has been successfully used in a variety of ligand screens such as identification of inhibitors of lipoxygenase activity (130).

1.4.2 Cell based assays

Cell free assays do not always reflect how a drug interacts with a target molecule within the complex environment of an intact cell. It has therefore been proposed that increased reliance on **cell based validation** early in the discovery process will prove economically advantageous (131).

An important advantage of a cell based assay comes from the following considerations. The probability of identifying a selective interaction between a compound and a biological target increases with the number of compound-target pairs assayed. This is the basic concept of empirical high throughput screening (HTS). Consequently, with respect to a single compound the number of biological targets available for screening determines the success. A cell is a complex biological system containing a huge number of target molecules that are all functionally connected: a high content bio-assay system. The cell reacts to the action of the compound by developing a distinct phenotype which is the assay read-out. Thus, cell based phenotypic screening is particularly effective in the search for bioactive compounds, although the target is not immediately apparent and needs to be identified subsequently.

This approach became feasible through the combination of high-throughput cellular assays and diverse libraries of compounds. The ability to perform genetic screens in cellular assays vastly increases the throughput – traditionally a key limitation when studying higher organisms. It also allows the separation of effects in somatic cells from those in development. This thus requires continued advances in combinatorial chemistry, protein biochemistry, miniaturization, automation, and global profiling technology. Trends in screening technologies include miniaturization (μHTS) to functional assays (cell based assays, CBA) to high content screening where the amount of data collected per well is much larger. Cell based assays have become indispensable to screen for targets that cannot be biochemically purified such as G-protein coupled receptors and ion channels.

1.4.2.1 Cell based assay: cell lines

In the transition from a simplified solution binding assay to *in vivo* testing, cell based testing has proven to be a valuable stepping-stone to quickly weed out toxic and non-functional compounds. The low cost and high speed of testing compounds in cell culture, and the obvious advantages of using intact cells as the most expedient first representation of the living patient, has made cell based testing a key component of drug discovery programs. Cell based testing is well established in drug discovery research with well-described cell lines and models such as the ones listed in the table below. The same physiological or disease conditions can be studied using cell lines from different organisms as can be seen in the table below.

Physiology/ Disease studied	**Cell line**	**Target molecule/ Pathway**	**References**
Alzheimer's	Drosophila S2 cells	Beta Secretase	(132)
	Rat primary cortical neurons	Beta Amyloid peptide (1-42)	(133)
	Human Embryonic Kidney HEK293 cells	Gamma Secretase	(134)
Cancer	Yeast KCT120a cells	Histone deacetylase	(135)
	Rat Chondrosarcoma (RCS) cells	Erk MAPK pathway	(136)
	Human ovarian carcinoma A2780 cells	MLH1 mismatch repair gene	(137)
Diabetes	Rat Hepatoma H4IIE cells	Glycogen Synthase Kinase 3 (GSK3)	(138)
	Chinese Hamster Ovary CHO- K1 cells	Beta catenin & GSK3	(139)
	Human Hepatoma HepG2 cells	Protein Tyrosine Phosphatase 1B	(140)
Intestinal Absorption	Human Intestinal Epithelial cells Caco2	Bile pigments	(141)
	Human Mast cells HMC-1	Peptidoglycan	(142)
Viral Replication	Monkey Kidney Epithelial Vero E6 cells	SARS virus replication	(143)
	Human Hepatoma HepAD38	Regulated replication of HBV	(144)

Table 1-5. Cell lines from different organisms used to study human diseases and physiology along with the molecule or pathway at which the cell based assays are targeted are listed.

1.4.2.2 Cell based assays: growth surface

A crucial parameter for cells growing attached to a surface is the surface itself which serves as a micro-environment. Hence while choosing a platform suitable for cell growth, it is essential to look at surface properties such as hydrophobicity and roughness and also possible cell surface interactions. Cellular attachment and spreading to display cellular morphology is also vital. Choice of an appropriate cell line to investigate and reproduce observed cellular phenotypes also plays a key role while deciding on surface suitability. The simple addition of serum to fibroblasts activates a genetic program that unfolds a complex tapestry of functional gene clusters activated in time (145). This program resembles, in part, that of the wound healing response, presumably because fibroblasts see serum *in vivo* only in the context of a wound. Growth surfaces such as polyamines may induce cellular differentiation of fibroblast-like cells. Polyamine coated growth surfaces facilitate primary neuron adhesion and differentiation.

Cell spreading and intercellular contact are the two physical cues that provide distinct inputs to regulate cell proliferation (146; 147). Using a specialised set of semiconductor manufacturing tools, cell culture substrates are micro-patterned with micrometer-scale islands coated with extracellular matrix ligands surrounded by non-adhesive regions. Culturing endothelial cells or hepatocytes on such substrates, single cells were made to attach on each island such that cells spread to the size and shape of the engineered islands. Cell spreading itself, in the absence of cell–cell contact and in constant growth factor concentrations, can thus switch cells between quiescence and proliferative states (148; 149). If pairs of cells are placed on such islands, such that cell–cell contact is introduced without changing cell spreading, contact induces proliferation (150).

Anderson et al. describe a method of **hit arrays** to identify the appropriate bio-polymer which could be used for biomaterial based therapies. These biomaterial microarrays were tested for their effects on human embryonic stem cells as well as their differentiation (151). The polymers were arrayed onto normal epoxy glass slides coated with poly hydroxy methacrylate (pHEMA) known to inhibit cell growth. This prevented the growth of cells in between the arrayed polymer spots. The microarrays they developed thus enabled both the rapid synthesis and cell based screening of biomaterial libraries. Bailey et al. developed a novel method of presenting small molecules impregnated in a **biodegradable polymer** (poly-(D),(L)-lactide glycolide copolymer, PLGA) to form small molecule microarrays,

which could be presented to cell based screening (152). Using this method they were able to screen for distinct p53 mediated cellular responses with respect to apoptosis.

1.4.2.3 Cell based assays: advantages

Cell based assays have a distinct advantage over protein or DNA based screening as this approach enables the screening of a complex cascade of events that result from signal transduction in a cell. CBA can exploit easy read-outs like phenotypic characteristics of the cells. Studies related to cancer usually focus on cell motility, proliferation and migration as important parameters on which to base the cell based assays. Cell death including apoptosis and necrosis are other parameters generally used as readout for such assays. Several optimised kits from companies such as Promega and Roche enable these assays to be carried out in almost any lab setting. Also, the cells used for these assays may be transformed, immortalised, or have certain genes knocked out or overexpressed in them. Breakthrough technologies such as Antisense and RNA interference make the applications of cell based assays endless. The ability to tag the cells with reporters for proteins also increases the types of parameters that can be assayed for and quantified.

Further specialised cell lines can be developed for specific tasks such as the HEK293/GFP2 beta arrestin cell line developed for quantification of G-Protein coupled receptors **(GPCR) assays** (153). Similarly **Cytoblots** are whole-cell immunodetection based assays that enable the monitoring of biosynthetic processes such as DNA synthesis and post- translational modifications (154). More specialised assays such as those for **ion channels**, associated with several neurological and muscular diseases (reviewed in (155)), require the use of modified cell lines such as clonal rat pituitary cell line GH4C1, possessing L-type voltage-operated calcium channels and Chinese hamster ovary cell line, stably expressing Kv1.3 potassium channel (156; 157). Similarly, the life span of fibroblast-like synoviocytes can be extended by transducing genes such as telomerase to facilitate the use of this cell line for CBA to study rheumatoid arthritis (158).

Another advantage of cell based assays is the construction of specific reporter cell lines that express the target protein of interest as a fluorescent or luminescent molecule that can be used for intensity based and phenotypic readouts. Changes in protein structure by events such as phosphorylation or cleavage lead to the change in its intracellular localization which

can be studied using fluorescent microscopes and is high throughput compatible using high content screening technology. Plasmids constructed with the protein and reporter of interest can be transfected into cells either chemically using readily available reagents such as Fugene or using electroporation. Table 1-6 lists examples of reporter cell lines constructed to enable studies related to specific proteins of interest.

Reporter	Assay	Cell line used	References
Chloramphenicol acetyl transferase	Viral versus mammalian promoter use in cell based assays, tissue specific regulation of insulin receptor gene	breast cell lines T47D and MCF-7, HepG2 and rat liver cells	(159; 160)
Beta Galactosidase	Detection of nuclear translocation of the glucocorticoid receptor	Chinese hamster ovary K1 cells	(161)
Secreted Alkaline phosphatase	human telomerase reverse transcriptase (hTERT) gene expression	Lung carcinoma cells H1299 and human foreskin fibroblasts hTERT-BJ1 cells	(162)
Human growth hormone	Secreted growth hormone levels	Mouse fibroblast A31 cells	(163)
Luciferase	Hypoxia response element VEGF	Human endothelial EAhy926 cells able to express luciferase in hypoxic conditions	(164)
Green fluorescent protein	Detect osteogenic differentiation upon stimulation	rat type I collagen promoter based construct Col1a1GFP stably transfected pre-osteoblastic MC3T3E1 cells	(165)

Table 1-6. Reporter cell lines used in cell based assays

1.5 Fluorescence reporter technologies

Assay volumes and the concomitant decrease in the use of biological reagents are perceived as key factors for the high throughput screening process. However, **assay miniaturization** is dependent on multiple factors, including the need for homogeneous assays, the ability to handle ultra-low volumes of liquids and the demand for sensitive readouts. Although, radiometric and absorbance-based techniques have been the traditional methods, there are clear limitations with respect to miniaturization. By contrast, the inherent efficiency of fluorescence readouts overcomes these boundaries, as a consequence of the multiple (typically many thousands) of excitation and emission cycles that each fluorophores molecule undergoes before photobleaching. Intrinsic scalability enables seamless miniaturization with fluorescence-based methods, right down to the single-molecule level. Fluorescence assays can deliver the same results, whether applied in 100 μl or 5 μl because readouts are, theoretically, volume-independent, although, in practice, the geometry of microtiter plates and detection equipment, such as imaging systems, restricts miniaturization for macroscopic measurements. In many cases, however, miniaturization using fluorescence readouts is only restricted by liquid handling, absorption or evaporation effects.

Green Fluorescent Protein (GFP) is a fluorescent molecule found in the jellyfish *Aequorea victoria*. Isolated in 1961 (166) and cloned in 1992 (167), it has become a popular reporter molecule for gene expression in biological systems. It exists as a 238-amino-acid protein; its crystal structure illustrates its conformation as a rigid β can, enclosing a helical region that contains the chromophore (168; 169). The chromophore itself is a cyclic tripeptide (Ser65-Tyr66- Gly67), and this region is required for fluorescence. Because of its compact structure, and because the chromophore is protected from the external environment inside the β can, GFP is extremely stable and resistant to bases, mild denaturing and reducing agents and high temperatures, and it is capable of renaturing after exposure to harsh conditions.

Several modifications of GFP have given rise to reporter molecules with superior monitoring capabilities compared to luciferase or colourimetric detection systems based on Horse radish Peroxidase (HRP) or Alkaline Phosphatase (AP). Wild type GFP is maximally excited in the near UV range 395 nm; with a minor excitation peak at 470 nm and emits green light at a 509 nm maximum with a shoulder at 540 nm. To improve detection in mammalian systems, several research groups have modified the sequence of GFP. One common variant, enhanced

green fluorescent protein (EGFP), contains two mutations in the chromophore region – Phe64 to Leu and Ser65 to Thr – that red-shift the spectral excitation peak to 489 nm. Red-shifted GFPs, such as EGFP, can be excited using the 488 nm argon-ion laser and can be detected using standard fluorescein (fluorescein isothiocyanate, FITC) filter sets used in both fluorescence microscopy and flow cytometry. Since the creation of red-shifted GFPs, further amino acid manipulations within the chromophore region have successfully produced colour shifts into the yellow and blue regions of the visible spectrum. The various variants of GFP and their applications have been reviewed in (170).

The different fluorescence-based methods used in cell free and cell based assays are as listed in table 1-7. These methods each exploit different aspects of fluorescence and present distinct advantages and disadvantages, particularly in terms of their sensitivity, robustness and susceptibility to artifacts. The underlying physical principles and applications of fluorescence in bioassays have been reviewed in more detail in (171-173). Fluorescent methods used in this thesis include GFP tagged recombinant proteins and Fluorescence Resonance Energy Transfer. Table 1-7 lists fluorescent technologies in use today in cell free and cell based systems. The various diverse fluorescence methods available provide diversity to the kinds of cell based assays that can be performed to achieve a more sensitive and reliable readout in high throughput assays.

Fluorescence Technology	Detection of	Use/ Advantage	References
Macroscopic fluorescence methods			
FP (fluorescence polarization)	rotation of single bio-molecules or their complexes in relation to their mass	Detection of cAMP in living cells	(174)
FLINT (fluorescence intensity)	Fluoro-substrate turnover	Direct enzyme assays	(175)
FRET (fluorescence resonance energy transfer)	Resonance energy transfer from a donor fluorescent molecule to an acceptor fluorescent molecule	Receptor activation, Identification of inhibitors	(176-178)
Single molecule fluorescence detection techniques			
FCS (Fluorescence correlation spectroscopy)	detection of single fluorescently labeled molecules in solution based on mass	Diagnosis of prion diseases	(179)
FLIM (Fluorescence lifetime imaging microscopy)	Imaging technique where lifetimes rather than intensity of signal is measured	Receptor inhibitor binding, dimer formation	(180; 181)
TRF (time-resolved fluorescence)	measuring fluorescent events usually from fluorophores with long lived signals, can be applied with FRET, FP etc	Inhibitor screening, measure interferon activity on human cells	(182; 183)
Single molecule FRET	Resonance energy transfer from a donor to an acceptor molecule both attached to the same biological molecule	Folding and unfolding of proteins	(184; 185)
FIDA (1D and 2D fluorescence intensity distribution analysis	Single molecule detection (based on mass as well as fluorescence intensity)	Screen for activators of p53, quantification of protein aggregates	(186; 187)

Table 1-7. Different fluorescence based screening methods applied to cell free and cell based systems

1.6 High throughput screening – limitations and advantages

The laborious **target validation step** usually presents the real bottleneck in high throughput screening; thereby reducing the pace with which new drugs are discovered today. The selective ligand-binding interaction validates the target, and inversely the binding to the target confirms the biological activity of the ligand. Inhibition of candidate gene function might not, in all instances, result in an obvious phenotypic change and, therefore, it might be relevant to first identify the proper biological context (with respect to specific signalling pathway the particular gene of interest functions). Recent developments have substantially improved the level of assay sensitivity and assay throughput, for studying gene function in cell based assays. Cell based assays will help elucidate the molecular changes that occur over time and that lead to the manifestation of a pathological phenotype.

A **successful cell based assay** relies on factors such as availability of large amounts of the same cell line, testing of cell lines from different tissues and organs, thereby introducing the feature of scalability and flexibility of the assay in question. Along with this aspect is raised the issue of standardisation of every aspect of the assay from cell maintenance to the quality of tissue culture or detection plates to the hardware used for HTS and the software used for data analysis, mining and storage. Moreover, assays relying on phenotypic analysis of cells can be largely influenced by changes in batches of cell culture media or reagents. The availability of these from reliable sources eases the HTS assay, making cell supply and recognition of this no longer a limiting factor. These are however expensive and so are the systems for detecting, analysing and documenting cell based assays, thereby generating the need for cost effective cell based assay options. The availability of kits and ready-made reagents for routine cell based assays based on cytotoxicity and proliferation not only increase the probability of cell based assays being used for preliminary screens but also appeal to labs where the focus of research may not be drug discovery.

The logistics of developing a high-throughput screen, delivering hundreds of thousands of compounds to the screen, screening the plates and producing possibly thousands of follow-up samples, requires an extensive infrastructure and involved planning, as well as time. Though the parameters of a successful HTS are now well established, one of the key factors remains **miniaturization** to 96, 384, 1536 well plates or even smaller formats without affecting the reproducibility, statistical significance of the results or robustness of the assay. The evolution of sensitive luminescent and fluorescent detection systems makes

miniaturization possible. Also the development of liquid handling robots makes it possible to screen for thousands of compounds at a time.

The parallel advances in biological tools to identify, mutate, express, characterise and purify proteins have also led to rapid identification of novel targets associated with disease processes. However, with this rapid progress another limitation has emerged in early phases of drug research: the need for processing large numbers of potential drug candidates and identifying those that have a high probability of becoming marketable products as early as possible. Screening a small molecule library in binding assays is usually the approach adopted by companies towards to identify possible lead drugs. Introduction of **databases** such as the **ChemMine**, **Cell Wall Mining** and **AgBase** aid in centralising the structure of compounds and information of their activity from a growing number of public providers and vendors of chemical screening libraries (188-190). Web accessible databases such as **PubChem**, an open, centralised database of small molecules serve as a chemical gateway to biomedical databases such as **PubMed**. More specialised databases such as **TarFisDock** identify targets with docking approaches (191). The **PDBbind**, **BindingDB** and **AffinDB** focus on the binding affinities between target and molecules (192-194).

1.7 The experimental concept

The various steps involved in generating an assay have been discussed in the previous section. The goal of this thesis is to develop a platform where cell based assays can be carried out on the same surface used for small molecule synthesis. This project aims at establishing a cell based assay to screen for the cleavage of human La protein. The "La"-assay will be screened against peptides and small molecules generated using the SPOT method explained in more detail in section 1.7.1. The basis of synthesis of small molecule compounds has already been discussed previously (section 1.2). The structure and function of the La protein, its interaction with the Hepatitis B Virus and current strategies against Hepatitis are reviewed below in sections 1.7.2 to 1.7.4.

1.7.1 SPOT synthesis technique

Arrays for screening the La protein will be generated with the SPOT-synthesis technique. This method belongs to a family of positional addressable solid phase methods that allows for the simultaneous parallel chemical synthesis on membrane supports (95). In principle,

dispensing of a small droplet of liquid on a planar surface of a porous membrane results in its absorption and formation of a circular spot. The SPOT synthesis technique relies on the formation of open reactors for chemical conversions where the reactive functions are anchored to the membrane support as in conventional solid phase synthesis (51). This method was first described by Dr. Ronald Frank and termed as SPOT synthesis (54; 95).

The SPOT technique is especially widely used for the generation of large repertoires of peptide fragments in a high density array format. In this case, low volatility solutions of N-terminally and side chain protected amino acids are distributed by manual or automated pipetting to defined positions on a modified cellulose membrane and are coupled *in situ* to the previously functionalised solid support. The basic concept came from observations made previously with combinatorial oligonucleotide (195) and peptide synthesis on separate, labelled membrane (cellulose) disks, indicating that chemical reactions can proceed to completion, when only enough reagent solution is used as can be taken up by the support material itself. This observation suggested that individual amino acid coupling reactions in a multiple parallel synthesis scheme could be carried out simultaneously on distinct areas of a continuous membrane sheet (95).

To prepare the membrane supports for chemical peptide synthesis, the entire cellulose membranes were originally modified by esterification with β-alanine (Fmoc-β-Ala) followed by the removal of the Fmoc protection group. Specially modified membranes for the preparation of either immobilised or solution phase peptides are available commercially. Special safety catch linkers have been developed that cleave also C-terminally unmodified peptide acids or amides directly into neutral aqueous buffer for direct use in bioassays. Fmoc-β-Ala is initially distributed on previously defined positions on the membrane generating sites for peptide synthesis. The remaining amino groups on the membrane are acetylated and therefore no longer accessible for further reactions. Elongation of the peptide sequence is an iterative process employing several washing steps as well as steps of de-protection, coupling and acetylation of amino acids. This is described in more detail in the Materials and Methods section 2.2.15.

The N-terminal protection groups are removed before distribution of different activated amino acids to different peptide sites. After the coupling reaction is completed, the remaining free amino groups are deactivated by acetylation. In the next step, the terminal protecting groups of the coupled amino acids are removed and the entire cycle can be

repeated. After coupling the last amino acid, all peptides are N-terminally acetylated to mimic the backbone of proteins. Although several steps during the SPOT peptide synthesis must be carried out manually, the delivery of the activated amino acids to the corresponding reaction sites has been automated using the SPOT synthesiser developed at ABIMED Analysen Technik GmbH (Langenfeld, Germany). The process automation helps to reduce the size of spots and thus increase the number of spots per area considerably. Currently, 2500 spots can be generated on a micro-titer plate sized sheet.

The SPOT-synthesis method opened up countless opportunities to synthesise and subsequently screen large numbers of synthetic peptides as well as other organic compounds arrayed on a planar cellulose support (95). They have become a very important tool for studying the protein-protein or protein-peptide interactions. Although SPOT-synthesis is not as impressively miniaturized as, e.g. the Affymax photolithographic technique (196), it fulfils similar demands with the advantage of a reliable and easy experimental procedure, inexpensive equipment needs and a highly flexible array and library formatting (94). The method permits rapid and highly parallel synthesis of huge numbers of peptides and peptide mixtures (pools) including a large variety of unnatural building blocks, as well as a growing range of other organic compounds. Further advantages are related to the easy adaptability to a wide range of assay and screening methods such as binding, enzymatic and cellular assays, which allow *in situ* screening of chemical libraries due to the special properties of the membrane supports. Therefore, peptide arrays prepared by the SPOT technique became quite popular tools for studying numerous aspects of molecular recognition, particularly in the field of molecular immunology. Much wider scope of their applications is reviewed in (197). Several examples of the application of the SPOT technology are as shown in table 1-8.

Application	Example
Identification of linear epitopes	Reviewed in (198)
Identification of paratopes	HIV antiviral activity (199)
Discontinuous epitope mapping	IL-10 (200) Bovine prion protein antibody mAb 15B3 (201)
Peptide antibody interaction	Transformation of L-peptide epitopes to D-peptide epitopes (202)
Protein - protein interactions	Characterisation of the proline rich binding motifs of the EVH1 domains in the Ena-VASP family (203)
Activity of cleaved solution phase peptides	Mapping T Cell epitopes (204; 205)
Enzyme substrate recognition	Protein kinase substrate specificity (206)
Animal immunization	(207)

Table 1-8. Applications of the SPOT technique

1.7.2 Introduction to the model La protein

An important attribute of the immune system is the ability to distinguish self from non self. When the immune system fails, an immune response is generated against cells and tissues of an individual's own body known as an auto-immune reaction. The ensuing auto immune tissue damage may be cell mediated as in Multiple Sclerosis or mainly mediated by the humoral immune response via autoreactive antibodies as is the case in Systemic Lupus Erythematosus or in Sjögren's syndrome. The La protein was first discovered as an autoantigen in Sjögren's syndrome (208; 209). Patients with Sjögren's syndrome, a systemic autoimmune rheumatic disease, frequently present autoantibodies to both organ- and non-organ-specific autoantigens. The most commonly detected autoantibodies are those directed against the ribonucleoproteins Ro/SSA and La/SSB. The presence of the antibodies in Sjögren's syndrome is associated with early disease onset, longer disease duration, parotid gland enlargement, higher frequency of extraglandular manifestations and more intense lymphocytic infiltration of the minor salivary glands.

Over the past several years, the structure and function of these autoantigens have been extensively studied. The human La protein is a 47 kDa nuclear phospho-protein known to be associated with all newly synthesised RNA polymerase III transcripts. It is ubiquitously expressed and there are about 2 x 10^7 molecules of the La protein per cell which is about 50 nM, making it as highly abundant in the cell as ribosomal protein (210; 211). The name La is derived from the name of the patient in which the antibody was detected, and SS-B refers to

the prevalence of the antibodies in Sjögren's syndrome. Although La was first characterised as a human protein, homologs have been identified in many eukaryotes ranging from yeasts and Drosophila to Xenopus (212-218). Within the nucleus, La is found to be both nucleoplasmic and nucleolar (219-222). However certain conditions such as apoptosis in mammalian cells may cause it to become cytoplasmic (223; 224).

1.7.2.1 The La Protein Function

The La protein belongs to a family of RNA binding proteins and is characterised by the presence of one or more RNA recognitions motifs (RRMs). La is associated with a very large number of nascent RNAs as well as viral encoded RNAs as listed below (Table 1-9). RNA chaperone activity is defined as an activity that either prevents RNAs from misfolding or that helps to open up RNA structures without ATP consumption (225; 226). The major role of La is to act as a molecular chaperone of newly synthesised RNAs by stabilising them from exonuclease digestion leading to either their nuclear retention or export and hence determining their correct fate (216; 227-231). In viruses, the La protein is required for viral replication and controls translation.

	Type of RNA interacting with the La protein	**Function of the La protein in the interaction**	**Reference**
Viral RNAs			
1	leader RNAs of several negative-strand viruses	Required for replication of viruses	(232; 233)
2	Adenovirus-encoded VA RNAI and VA RNAII	Formation of a ribonucleoprotein (RNP) particle	(234; 235)
3	Dengue Virus RNA	Chaperon to facilitate replication	(236)
4	the Epstein-Barr virus–encoded EBER 1 and EBER 2 RNAs	Chaperone activity	(237; 238)
5	Hepatitis B Virus RNA	Stabilization	(239)
6	Hepatitis C Virus RNA	Translational control	(240)
7	Herpes simplex virus type 1	facilitates viral replication in transfected mouse 3T3 cells	(241)
8	HIV type I virus	Translational control	(242)
9	Norwalk Virus RNA	Translation/ Replication	(243)
10	Polio virus m RNA	Translational control	(244)

Other RNAs			
1	Bip RNA	Translational control	(245)
2	Histone RNA	Stabilization	(246)
3	pre-5sRNAs	Maturation of pre-5sRNAs	(247)
4	pre-tRNAs	maturation and efficient folding of pre tRNAs	(248-251)
5	Telomerase RNA	Chaperone for Telomerase RNP binding	(252)
6	TOP mRNA	Translation	(253)
7	RNAs transcribed by RNA polymerase II such as U1, U2, U4, and U5 pre-snRNAs	La interaction protects against exonuclease digestion	(254; 255)
8	U6 small nuclear RNAs	Binds to precursors of U6 snRNAs and aids in their maturation	(256)
9	Vault RNA	Chaperone for the vault RNA	(257)
10	Y RNAs	La binding contributes to nuclear retention	(258)

Table 1-9. Interaction of the La protein with different RNAs

1.7.2.2 The La Protein Structure

The **N terminus** of La is well conserved from yeast to humans, whereas the human La protein gained an additional C-terminal domain absent in the yeast Lhp1p. The N terminus of all known La proteins contains the 60–amino acid **La motif** [previously called the La domain], a highly conserved sequence that is also present in a number of otherwise unrelated proteins. Recent structural data revealed that the N-terminal La motif does not adopt an RRM structure but folds into a **winged helix motif** (WHM), whereas the central and C-terminal RRMs (RRMs 1 and 2, respectively) resemble **RNA recognition motifs** (RRM). The ribonucleoprotein RNP2 signature of the RRM1 is known to be of general importance for RNA binding.

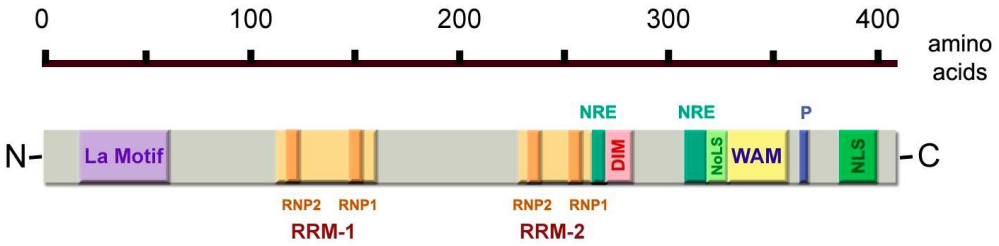

Figure 1-3. Schematic diagram depicting the various motifs in the La protein.
The human La protein contains a winged helix La motif, central and C terminal RNA recognition Motifs (RRM) with internal conserved ribonucleoprotein (RNP) signatures, nuclear localisation (NLS), nucleolar localisation (NoLS) and nuclear retention signals (NRE). A dimerization/multimerization signal and a Walker A motif are also present along with the Ser 366 phosphorylation site essential for RNA interactions.

The **C-terminal domain** is the least conserved part of the La protein, varying in both size and sequence between species. This portion of the La protein (defined as being C-terminal to the central RRM), usually contains between 40% and 50% charged residues. The C-terminal region is quite variable in length, ranging from 70 amino acids in the yeasts *S. cerevisiae* and *S. pombe* to more than 220 amino acids in humans and *Xenopus laevis*. The lower degree of conservation of the C-terminal portion is emphasised by the finding that almost all differences between the human and mouse proteins are found in the C terminus. At least some of the additional size of vertebrate La proteins comes from a potential atypical RRM in this region. A possible Walker A motif, a nucleoside triphosphate–binding sequence found in many ATP-binding proteins, has also been noted in the C terminus of vertebrate La proteins. Trafficking signals in the human La include a nuclear localization signal (NLS), a nuclear retention element (NRE) and a nucleolar localization signal. In addition, human La autoantigen (hLa) also contains a dimerization domain as well as a conserved basic region. Phosphorylation events, in particular those responsible for communication signals, are among the most common post-translational protein modifications that are necessary for cell functions. In human cells, most La is phosphorylated on serine-366 by protein kinase CK2, resides in the nucleoplasm, and is associated with nascent pre-tRNAs. Non-phosphorylated La is mostly concentrated in the nucleolus and was independently found at tRNA and other RNA polymerase III-transcribed genes, but it also resides in the cytoplasm associated with 5′ TOP mRNAs that encode ribosomal proteins and translation factors.

1.7.3 The HBV RNA and its interaction with the La protein

HBV is a noncytopathic, enveloped, double-stranded DNA virus that causes acute and chronic hepatitis and hepatocellular carcinoma (HCC). It replicates through a RNA intermediate and encodes four unspliced, overlapping messages that terminate at a common polyadenylation signal. HBV is transmitted sexually and from mother to infant at birth like the human immunodeficiency virus (HIV). More than 2 billion people alive today have been infected by HBV, and more than 350 million people are chronically infected (259). The chronic infection may lead to liver cirrhosis and liver cancer. Accordingly, HBV causes approximately 1 million deaths each year worldwide.

Viral hepatitis is initiated by an antigen-specific antiviral cellular immune response. Although clearance of most virus infections is widely thought to reflect the killing of infected cells by virus specific T cells, recent data also suggest that non-cytolytic intracellular viral inactivation by certain inflammatory cytokines released by activated lympho-mononuclear cells may play an important role in the clearance of at least some of these viruses from the infected cell (260). This is also true for HBV. Inflammatory cytokines, such as gamma interferon (IFN-γ) and tumour necrosis factor alpha (TNF-α), can abolish hepatic HBV gene expression and replication in the livers of these animals where HBV-specific cytotoxic T lymphocytes (CTLs) are transferred. The cytokines destabilise HBV mRNA thereby suppress HBV gene expression by a posttranscriptional mechanism.

In HBV transgenic mice injected with CTLs and infected with lymphocytic choriomeningitis virus and murine cytomegalovirus, the liver nuclear extracts contain three proteins (p45, p39 and p26) that bind a 91-nucleotide (nt) in vitro transcript of HBV. All the three proteins p45, p39, and p26 are recognised by anti- La antibodies and bind HBV RNA in a phosphorylation-dependent manner. The La autoantigen (p45) and La fragments (p39 and p26) are HBV RNA-binding proteins, which bind to a predicted stem-loop structure located between nucleotides 1243 and 1333 of HBV RNA (numbering according Galibert et al.). A tight correlation was observed among the downregulation of HBV RNA, the disappearance of p45, and the appearance of p26, suggesting that these proteins might contribute to the regulation of HBV mRNA stability by the cytokines.

The La protein, especially p45, may be part of a complex mechanism that controls HBV RNA stability, constitutively and in response to inflammatory cytokines (261). The La protein interacts with a small cis-acting element located within the viral RNA between nt 1275 and

1291. The tight temporal correlation between the cytokine-mediated downregulation of HBV RNA and the cytokine-induced fragmentation of full-length La led to the assumption that full-length La stabilises HBV RNA by interacting with the cis-acting element. HBV RNA is more accessible to endoribonucleolytic cleavage after the disappearance of full-length La protein. The presence of full length La protein correlated directly with the presence of HBV RNA, detectable when the viral RNA was abundant and disappearing when the RNA degradation was post transcriptionally induced in response to IFN-γ and TNF-α. Figure 1-4A shows the presence and binding of full length La protein in control saline conditions to HBV RNA and its disappearance on CTL injection. Figure 1-4B shows the predicted region of the HBV RNA that binds to the La protein (HBV RNA.B).

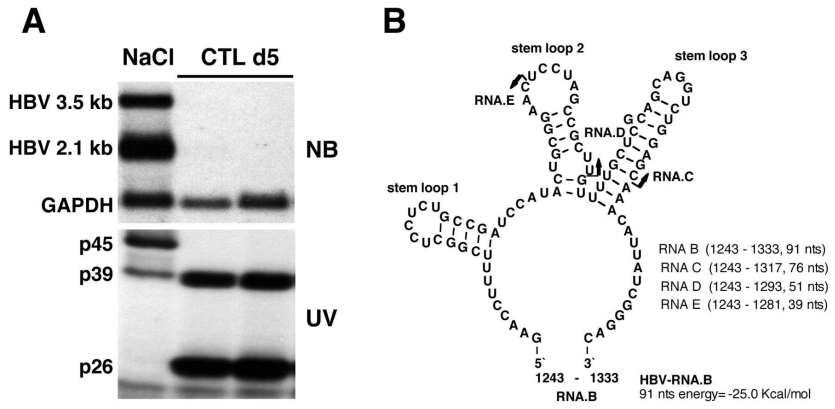

Figure 1-4. HBV RNA-binding proteins p45 and p39 are detectable in liver nuclear extracts from NaCl-injected mice, while p39 and p26 are detectable in CTL-injected mice. (adapted from (261))
(A) Northern blotting and UV cross-linking analysis of 20 mg of total liver RNA or 5 mg of liver nuclear extract prepared from the same liver were performed. Sex and serum HBsAg-matched mice (lineage 1.3.32) were intravenously injected with 10^7 CTLs or with saline and sacrificed on day 5 after CTL administration. The upper panel shows the Northern blot analysis, and the lower panel shows the UV cross-linking analysis of nuclear extracts.
(B) Predicted secondary structure of HBV in vitro transcript RNA.B used in this study. The secondary structure was calculated with the program MFOLD version 3 by Zuker and Turner available on the MFOLD server. Arrows indicate the 39 ends of in vitro transcripts RNA.C and RNA.D and of an oligoribonucleotide, RNA.E. The positions for all RNAs are shown according to the HBV *ayw* subtype sequence.

1.7.4 Drug Strategies against Hepatitis

Hepatitis B vaccines are of two types, plasma derived and recombinant. **Recombinant vaccines** are produced by cloning the gene encoding HBsAg into yeast cells and are

increasingly replacing plasma derived vaccines. Infection with hepatitis B virus (HBV) can be prevented by vaccination with the major HBV surface antigen (HBsAg) (262; 263). However, HBV mutants are able to escape immune responses against hepatitis B vaccines and to establish chronic infection in a liver transplant recipient. These HBV mutants carry multiple amino acid substitutions around and within the HBsAg thereby exhibiting a reduced reactivity to a panel of anti-HBs antibodies (264). Three **drugs**—interferon alfa, lamivudine, and adefovir—are approved in several countries for use in chronic hepatitis B (265). Of these, interferon has both antiviral and immunomodulatory activity; lamivudine and adefovir are primarily antiviral. Emtricitabine, entecavir, telbuvidine and clevudine are currently under investigation.

Interferon alfa, a host cytokine produced in response to any viral invasion, has immunomodulatory, antiviral, and anti-fibrotic properties. It was first used in the 1980s and was the first drug to be found useful in the treatment of chronic hepatitis B. However, interferon has many adverse effects. **Lamivudine**, a synthetic nucleoside (cytosine) analogue available since 1998, undergoes intracellular phosphorylation to its active metabolite lamivudine triphosphate and inhibits viral reverse transcriptase, causing premature chain termination during viral DNA synthesis. **Adefovir dipivoxil**, a nucleotide analogue of deoxyadenosine monophosphate, inhibits viral reverse transcriptase activity in hepatitis B virus. However, some mutants of hepatitis B virus also show Adefovir resistance. Hence, there is a need for newer approaches to hepatitis vaccines as well as drugs available for hepatitis B. The use of **RNA interference** in the treatment of viral hepatitis is an emerging strategy in the treatment of this disease (266). RNA interference (RNAi) is a mechanism of gene regulation in plants, invertebrates and, more recently, in mammalian cells in which target mRNAs are degraded in a sequence-specific manner. RNAi strategies against HBV have been targeted against viral RNA replication in cells (267; 268) as well as in mice (269; 270). There is thus still a need for newer strategies against HBV and this is the exploited in this thesis.

1.8 Aims of this thesis

AIM 1: To develop a platform where small molecule synthesis and cell based assays can be carried out on the same surface.

The main goal of this thesis is to establish a novel chemical platform that is suitable for cell based assays. Several platforms from membranes to slides have been discussed in section 1.3. In this thesis the aim is to develop a platform where cell based assays can be carried out on the same surface after chemical synthesis. The patches used for the cell based assays are shown in figure 1-5. Optimization of the polypropylene surface for a) cell growth and viability b) cell morphology, phenotypes and c) chemical manipulation of phenotypes will be carried out.

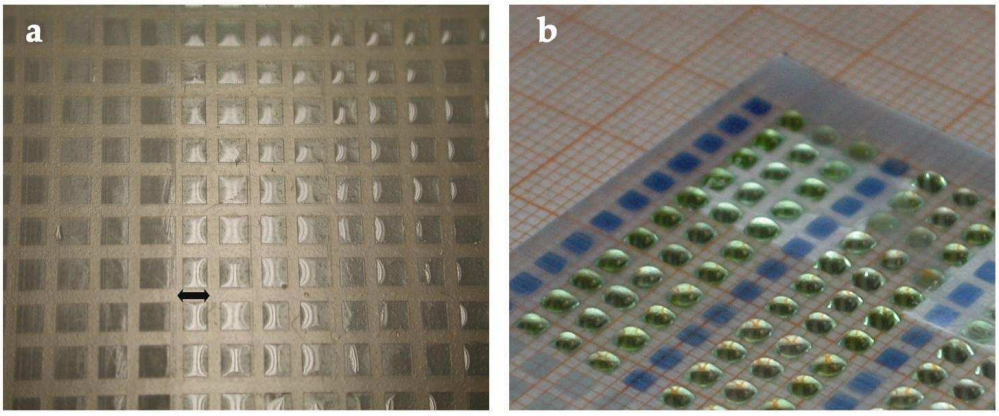

Figure 1-5. Poly-propylene foils used in cell based screening
The above panels depict the PP foils (Patches) used in the cell based screenings done in this thesis. Panel a depicts the array of patches on the PP foil. The black arrow depicts the side of each a patch square having dimensions of 2mm. Panel b represents organic medium spots pipetted onto the individual patches. In case of cell based assays, cells in cell culture media were pipetted onto patches with modified surface chemistry.

For this, cell lines will first be grown on polypropylene foils with chemical modifications in order to test their ability to sustain cell growth. Once this has been established, the patches will be tested for replication of simple cellular phenotypes such as cytokinesis. Further, chemical modification of cellular phenotypes in response to small molecules synthesised on the surface will also be tested. With the establishment of such a platform it will be possible to screen for libraries synthesised on the platform using cell based assays with the model La protein.

<u>AIM 2:</u> Development of a cell based assay to be applied to the new screening platform in order to screen for small molecule compounds which induce intracellular cleavage of the La protein.

Simultaneously for a more functional assay, the model La protein whose proteolytic cleavage can be targeted as a drug strategy against Hepatitis, will be used to develop cell based screens that can be applied to the patches. It is known that inhibition of the human La protein by RNA interference down regulates hepatitis B virus mRNA in 2.2.15 cells (271). Also, HBV RNA is more accessible to endoribonucleolytic cleavage after the disappearance of full-length La protein. The presence of full length La protein correlated directly with the presence of HBV RNA, detectable when the viral RNA was abundant and disappearing when the RNA degradation was post transcriptionally induced in response to IFN-γ and TNF-α.

Peptide or small molecule libraries can thus be screened using cell based assays based on La protein binding/cleavage strategy. For this a FRET La molecule will be developed by the group of Dr. Tilman Heise at the HPI in Hamburg.

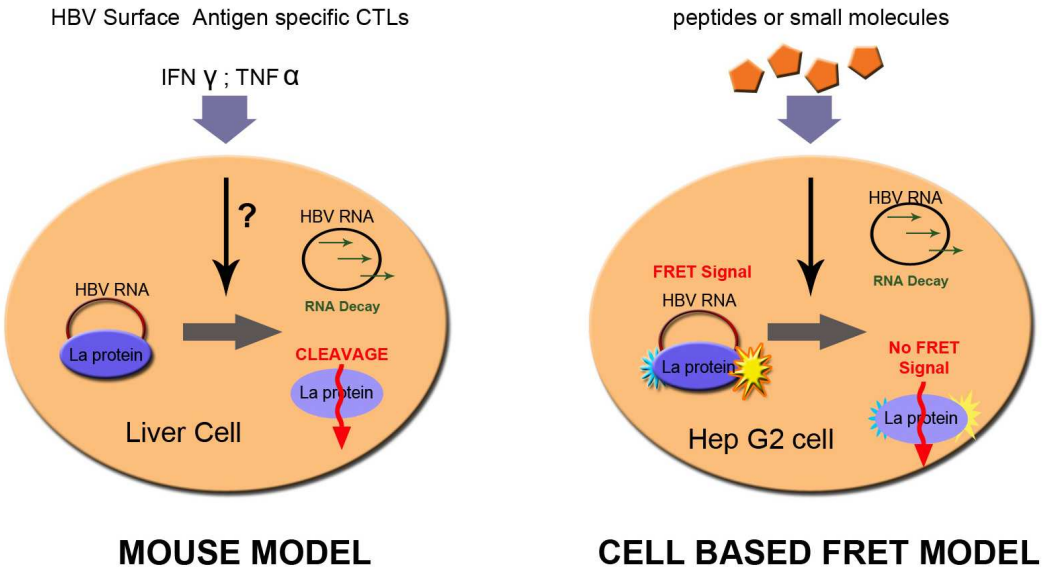

Figure 1-6. Schematic representation of the development of cell based assays using a human FRET-La protein.

In the mouse model, inflammatory cytokines, such as gamma interferon (IFN-γ) and tumour necrosis factor alpha (TNF-α) are known to abolish hepatic HBV gene expression and replication in the livers

of mice where HBV-specific cytotoxic T lymphocytes (CTLs) are transferred. The cytokines destabilise HBV mRNA thereby suppress HBV gene expression by a posttranscriptional mechanism. The presence of full length La protein correlated directly with the presence of HBV RNA, detectable when the viral RNA was abundant and disappearing when the RNA degradation was post transcriptionally induced in response to IFN-γ and TNF-α. This has been schematically illustrated in the left panel. In the cell based model, the FRET La construct on being transfected into cells such as HepG2 can be used to screen for peptides and other small molecules that can activate protease that can cleave the La protein. This screening for small molecule compounds would be based on the disappearance of the FRET signal from the FRET La construct.

<u>AIM 3:</u> Screen random SPOT libraries to identify peptides that can bind to the Wildtype La protein.

In order to validate the assay concept and cellular constructs, a positive control would be highly desirable. Therefore SPOT peptide libraries will be screened for the identification of random peptides that can bind to the wildtype La protein that could be used to competitively inhibit the binding of the La protein to the HBV RNA. In 2002, Horke et al. published several mutants of the La protein, one of which does not bind to the HBV RNA. This mutant (Δ2) could be used to screen for peptides that bind specifically to the wildtype La protein but not to the mutant La protein as is illustrated in the diagram below (Figure 1-7).

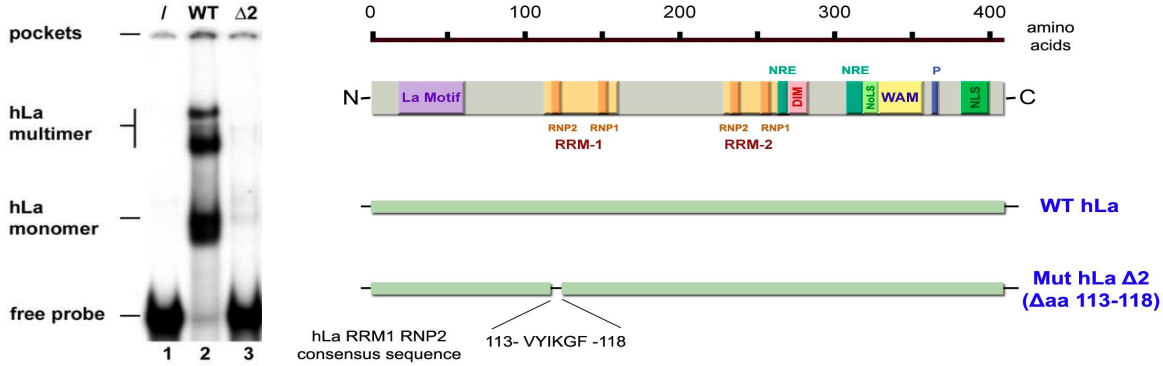

Figure 1-7. Wildtype La and Mutant La (Δ2) used for screening SPOT peptide libraries.
(A)Standard gel retardation assay to show that the interaction between hLa and HBV RNA.B strongly depends on the RNP-2 signature of RRM-1. Wild type recombinant hLa (WT, lane 2) and recombinant hLa-Δ2 (lane 3) with a deleted hLa RNP-2 signature (Δ2, aa 113–119) of RRM-1. was analyzed for HBV RNA.B binding. Lane 1, reaction without hLa. (from (272-274), published data).
(B)Schematic representation depicting the region and six amino acid sequence of the RNP2 of RRM1 that is deleted in the hLa Δ2 mutant. For a more detailed description of the wildtype La protein domains, refer to figure 1-3.

For this a radioactive strategy will be used as depicted schematically in the figure 1-7. An *in vitro* transcription/translation system will be used to synthesise radioactive recombinant wildtype and mutant (Δ2) La proteins to screen for peptides by carrying out affinity binding assays with random peptide libraries. Those peptides that bind to Wildtype La and not to Mutant La will be further validated in the cell based assay of AIM 2. The peptide libraries for screening will be presented as high density arrays on membrane supports manufactured by SPOT synthesis. Hexa- and octapeptide libraries in the form of arrays of XX12XX and XXX12XXX pools (X = random position occupied by all 20 amino acid residues; 1,2 defined position occupied by only one amino acid residue) will be applied.

As outlined in the schematic figure 1-8, the wildtype La binds to 2 peptide spots (green) that are not bound by the mutant La protein using the same peptide library, thus yielding two potential peptides based on which the next set of libraries can be synthesised.

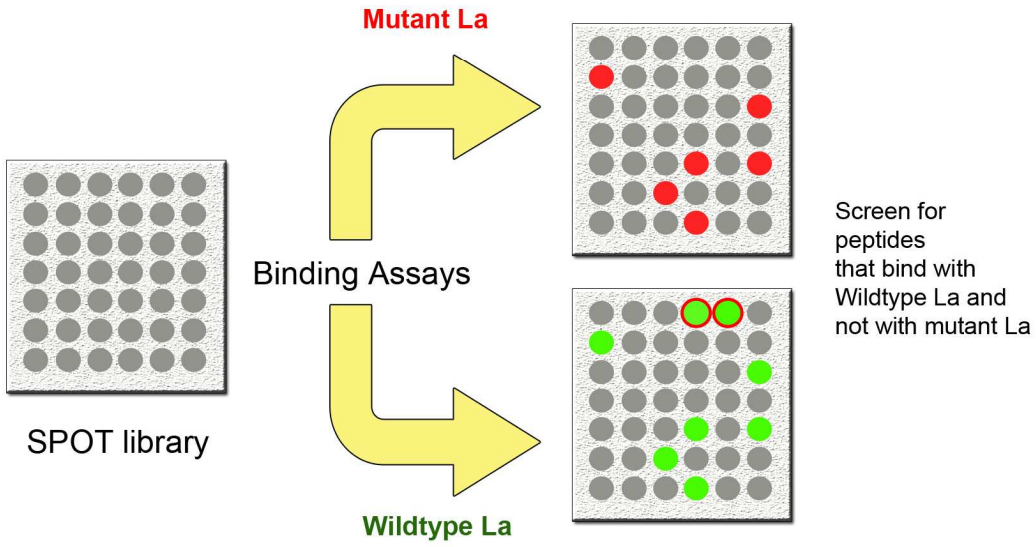

Figure 1-8. Schematic representation of *in vitro* SPOT library binding assays using recombinant wildtype La or mutant La proteins.
Binding assays with *in vitro* synthesised radioactive wildtype or mutant La protein will yield spots that can bind with the wildtype La protein but not the mutant La protein. Since the only difference between the two proteins is their ability to bind to the HBV RNA, screenings of such peptide libraries will allow the identification of peptides that require the HBV RNA binding site to bind to the wildtype La protein.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used during the course of this work were of analytical grade. All buffers containing water were made with autoclaved Millipore water. All the chemicals not mentioned here were from Merck, Roche, Carl Roth or Fluka.

Acrylamide/Bisacrylamide (40,29:1)	Carl Roth
Agarose	Peqlab
Bacto tryptone	BD Biosciences
Bacto yeast Extract	BD Biosciences
Bovine Serum Albumin	Sigma
DEPC	Sigma
DMEM	Gibco
DMSO	Sigma
EDTA	Sigma
Ethidium bromide	Sigma
FBS	Gibco
Glutaraldehyde	Sigma
Glycerol Ultrapure	Invitrogen
Gylcine	Sigma
Igepal CA630	Sigma
Imidazole	Sigma
IPTG	Carl Roth
K_3FeCN_6	Merck
L-Arginine	Sigma
Methanol	Mallinckrodt Baker
Mineral oil	Carl Zeiss
Mowiol	Calbiochem
NaCl	Carl Roth
Sodium dodecyl sulphate (SDS)	Carl Roth
NaH_2PO_4	Calbiochem, Merck
Ni-NTA-Agarose	Qiagen
NP-40	Sigma
n-propyl gallate	Sigma
Nuclease free water	MBI Fermentas
Paraformaldehyde	Fluka & Riedel
Poly-L-Lysine	Sigma

Protease Inhibitor Cocktail	Roche
SDS 10% solution	Sigma
Silver Nitrate	Merck
Skim Milk	Difco
TEMED	Roth
Trizma base	Sigma
Triton X-100	Roche
Tween-20	Sigma
Xylencyanol	Fluka & Riedel

2.1.2 General buffers and solutions:

Cell Freezing Medium:
Cell culture Media plus 10% DMSO

La protein binding buffer:
10 mM Tris HCL pH 7.4, 150 mM NaCl, 3 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.5 % NP-40

PBS buffer (10X):
80 g NaCl, 2 g KCl, 14.4 g $Na_2HPO_4.2H_2O$, 2.4 g KH_2PO4, H_20 to 1L, pH 7.4 with 1 M HCl and autoclave

Complete Protease-Inhibitor (100X, Roche):
2 tablets in 1 ml nuclease free sterile water

TBS (10X):
30 g Tris base, 80 g NaCl, 2 g KCl, H_2O to 1L, pH 7.4 with 1 M HCl and autoclave

TBST Buffer:
1X TBS plus 0.05% (v/v) Tween 20

2.1.3 DNA/protein markers, restriction enzymes, buffers, proteases and inhibitors

Alkaline Phosphatase	Roche
Alkaline Phosphatase, 10x buffer	Roche
Chymotrypsin	Sigma
Complete protease inhibitor	Roche
100 bp DNA Ladder, 100-1517 bp	New England Biolabs
1 kb DNA Ladder, 500-10.000 bp	New England Biolabs
Smart Ladder, 200-10,000 bp	Eurogentec
DNase, RNase-free	Promega
dNTPs	Promega

Elastase	Sigma
Lysozyme	Serva
Proteinase K	Sigma
Prestained broad-range Marker	New England Biolabs
Restrictions enzyme EcoR I	New England Biolabs
Restrictions enzyme Hind III	New England Biolabs
Restriction enzyme Xho I	New England Biolabs
dNTPs	Promega
Shrimp-alkaline Phosphatase	Roche
Shrimp-alkaline Phosphatase, 10X buffer	Roche
T4 Polynucleotide Kinase	Roche
T4 Polynucleotide Kinase, 10X buffer	Roche
Taq-DNA-Polymerase	Invitrogen
Taq-DNA-Polymerase, 10X buffer	Invitrogen
Thrombin	Sigma

Antibiotics and radioactivity

Kanamycin Stock: 10 mg/ml stock in water, sterile filtered, working conc. 50 μg/ml	Sigma
Ampicillin Stock: 50 mg/ml in water sterile filtered, working conc. 60 μg/ml	Sigma

Radioactivity:

S^{35} Methionine (GE Healthcare Europe GmbH)	Amersham Biosciences

2.1.4 Materials

Amicon-YM10 Concentrators	Millipore
Dialysis membranes:	
- Spectra/ Por MWCO 6,000-8,000	Spectrum Europe BV
- Zellu Trans MWCO 12,000 to 14, 000	Carl Roth
Eppendorf tubes	Eppendorf
G25-spin columns	Amersham Biosciences (GE Healthcare Europe GmbH)
GF/C-round filter	Whatman
Ni-NTA spin columns	Qiagen
Nitro-cellulose membranes (GE Healthcare Europe GmbH)	Amersham Biosciences
X-Ray-Film	Kodak BioMax XAR Film
Whatman Paper, 3mm	Whatman
Cell culture Dishes and flasks	Greiner bio-one

2.1.5 Kits and ready to use reagents

Amido black staining solution	Sigma
In vitro protein transcription & translation	Promega
Bradford protein assay reagent	Bio-Rad
DNA purification Maxi/-Mini kits	Qiagen
FuGene-6 transfection reagent	Roche
Ni-NTA spin columns and reagents	Qiagen
QIAquick Gel extraction kit	Qiagen
QIAquick PCR purification kit	Qiagen
Rapid DNA ligation kit	Roche
Red Alert western blot stain	Novagen
Enhanced Chemiluminescence Western Blotting detection reagents	Amersham Biosciences (GE Healthcare Europe GmbH)

2.1.6 Instruments

Agarose gel tanks

Agagel Mini, Agagel G45	Biometra
Agagel Maxi	Biometra

Power Supply

High Voltage Power pack P30	Biometra
Minicell Power pack	Biometra

Gel documentation

CCD Camera UVT-20 and EASY UV Transilluminator	Herolab

Protein Gel tank

Mini -Protean 3 cell	Biorad

Western Blotting Apparatus

Semi-dry Fast blot apparatus B 33	Biometra

Centrifuges

Sorvall centrifuge	Kendro Laboratory Products
Sorvall rotors: SS-34, SLA-1500, SLA-3000	
Cell culture centrifuge	Eppendorf

Photometers

GeneQuant II, RNA/DNA calculator	Pharmacia Biotech Europe GmbH
Ultrospec 2000 (for bacteria)	
UV/Visible Spectrophotometer	Pharmacia Biotech Europe GmbH

Microscopes

Confocal fluorescence microscope	Zeiss Axiovert, Carl Zeiss
Laser scanning fluorescence microscope	LSM 510 meta, with meta detector, Carl Zeiss

Lenses

Plan Neo 40X	Carl Zeiss
Plan ApoChr 33	Carl Zeiss
Achroplan LD 40X, 20X	Carl Zeiss

Other equipments

UV Crosslinker, Stratalinker 1800	Stratagene
pH meter, CG 840	Schott-Geräte GmbH
Thermomixer, 54 36	Eppendorf
Incubator, Multitron	Infros HT GmbH
Shakers, Rotomax120, Duomax120	Heidolph Instruments GmbH
Weighing balance, TE 1535 and R160P	Sartorius
Phosphor imager Fujifilm BAS 2500	Fuji Photo Film (Europe) GmbH,
Agfa Curix 60 film developer	Agfa-Gevaert Group

2.1.7 Biological materials

Genotype of bacterial strains used from Invitrogen

Escherichia Coli DH5 α
F$^{-}\phi$80dlacZΔM15 Δ (lacZYA-argF)U169 deoR, recA1 endA1 hsdR17 (r_{K}^{-} m_{K}^{+} phoA supE44 λ^{-} thi-1 gyrA96 relA1

Escherichia Coli BL 21 (DE3)
F - *ompT hsd*SB (rB - mB -) *gal dcm rne131* (DE3)

Mammalian Cell Lines used in this thesis and their sources

3Y1:	Rat embryonic fibroblasts	from Dr. K. Rottner, GBF
A431:	Human epidermoid cancer cells	from Dr. F. Sasse, GBF
A498:	Human kidney cells	from Dr. F. Sasse, GBF
A549:	Lung carcinoma cells	from Dr. F. Sasse, GBF
HeLa:	Human cervical carcinoma cells	from Dr. T. Heise, HPI Hamburg
Huh7:	Human Hepatoma cells	from Dr. T. Heise, HPI Hamburg

HepG2:	Human Hepatocellular carcinoma	from Dr. T. Heise, HPI Hamburg
L929:	Mouse fibroblasts	from Dr. F. Sasse, GBF
PC3:	Human prostate cancer cells	from Dr. F. Sasse, GBF
PtK2:	Rat kangaroo kidney epithelium	from Dr. F. Sasse, GBF
SKOV3:	Ovarian carcinoma cells	from Dr. F. Sasse, GBF

Vectors and engineered plasmids used in this thesis

1. pET 28b(+) human La –WT **(pET -huLa -WT)** described in Horke S. et al. in 2002 (272).
2. pET 28b(+) human La –Mut Δ2 **(pET -huLa -MutΔ2**) described in Horke S. et al. in 2002 (272).
3. pEGFP -La -WT described in Horke et al. in 2004 (273).
4. pEGFP -La -WT rev2xNES(+) constructed from the pEGFP -La -WT plasmid by adding a Nuclear Export Signal (NES).
5. pcDNA 3.1 (-)-La-Cygnet-2 **(Cygnet La)** constructed from the Cygnet plasmid as described in Honda et al. in 2001 (275) and insertion of the WT hLa gene in place of the PKG gene
6. pEYFP-C1 vector from Clontech
7. pECFP-C1 vector from Clontech

Antibodies

Anti -La antibody in mouse, 4B6	from HPI, Hamburg
Anti -GFP antibody, A. V. MAb JL -8	BD Biosciences
HRP conjugated secondary antibody goat anti-mouse	Dianova
Anti-tubulin antibody	Sigma

Primers for cloning of pEGFP -La -WT NES (+):

NES of the Protein kinase inhibitor (PKI), judged as a fast NES (5±10min)
PKI-S:
5′-T C*GA GCT C*AA TTA GCC TTG AAA TTA GCA GGT CTT GAT ATC AAC TC-3′
PKI-AS:
3′- *CGA G*TT AAT CGG AAC TTT AAT CGT CCA GAA CTA TAG TTG AGA GCT-5′

NES of HIV-1 Rev, judged as a medium type 1 NES (10±20min)
REV-S:
5′-T C*GA GCT C*AA CTT CAG CTA CCA CCG CTT GAG AGA CTT ACT CTT TC-3′
REV-AS:
3′- *CGA G*TT GAA GTC GAT GGT GGC GAA CTC TCT GAA TGA GAA AGA GCT-5′

NES of Adenovirus type 5 E1B-55K, judged as a slow NES (30±60min)
E1B-S:
5′-T C*GA GCT C*AA CTG TAT CCA GAA CTG AGA CGC ATT TTG ACA ATT TC-3′
E1B-AS:
3′- CGA GTT GAC ATA GGT CTT GAC TCT GCG TAA AAC TGT TAA AGA GCT-5′

2.1.8 Software

CCD Camera	EASY Image 3.16 software
Confocal fluorescence microscope	IP Lab spectrum, version 3.2
Image processing software	Adobe photoshop, version 7
LSM 510 meta	LSM 510 software, version 3.2.SP2
Protein estimation	TECAN Magellan, versions 2 and 3
Fluorometer, FLUOROMAX 3	Instrument Control Center, version 2.2.12 B
Zeiss Microscope	Axiovision, version 3.1 and 3.0
Image Reader LAS 1000	Image Reader LAS 1000 Pro, version 2.5
Phosphor imager Fujifilm BAS 2500	AIDA version 3.51
Spot binding assays analysis	AIDA version 3.51

2.1.9 Providers

Abimed Analysen Technik	Langenfeld, Germany
Agfa-Gevaert Group	Cologne, Germany
Amersham Biosciences/ GE Healthcare Europe GmbH	Munich, Germany
BD Biosciences	Heidelberg, Germany
Biochrom Ltd.	Cambridge, UK
Biometra Biomedizinische Analytik GmbH	Göttingen, Germany
Biorad Laboratories GmbH	Munich, Germany
Calbiochem	Merck KGaA, Darmstadt, Germany
Carl Roth GmbH	Karlsruhe, Germany
Carl Zeiss MicroImaging GmbH	Goettingen, Germany
Dianova	Hamburg, Germany
Difco	OttoNordwald KG, Hamburg, Germany
Eppendorf	Hamburg, Germany
Eurogentec GmbH	Köln, Germany
Fluka & Reidel	Sigma, Steinheim, Germany
Fuji Photo Film (Europe) GmbH	Duesseldorf, Germany
Grenier bio-one	Frickenhausen, Germany
Heidolph Instruments GmbH & Co. KG	Schwabach, Germany
Herolab	Wiesloch, Germany
Infros HT GmbH	Einsbach, Germany
Invitrogen	Karlsruhe, Germany
Kendro Lab Products	Bad Homburg, Germany
Mallinckrodt Baker, Deutschland	Griesheim, Germany
Merck KGaA	Darmstadt, Germany
MBI Fermentas	St. Leon-Rot, Germany
Millipore	Eschborn, Germany
New England Biolabs	Frankfurt am Main, Germany
Novagen	Merck KgaA, Darmstadt, Germany
Pharmacia Biotech Europe GmbH	Freiburg, Germany
peqLab Biotechnologie GmbH	Erlangen, Germany
Promega GmbH	Mannheim, Germany

Qiagen	Hilden, Germany
Roche Diagnostics GmbH	Mannheim, Germany
Schott-Geräte GmbH	Hofheim, Germany
Sigma	Steinheim, Germany
Spectrum Europe BV	Breda, the Netherlands
Stratagene	La Jolla, CA, USA
Whatman GmbH	Dassel, Germany

2.2 Methods

2.2.1 Growth of bacteria: transformation

LB medium:

For 1 L Bacto tryptone 10 g, Yeast extract 5 g, NaCl 10 g, pH 7.0 with 5 N NaOH

LB agar:

For 1 L Bacto tryptone 10 g, Yeast extract 5 g, NaCl 10 g, Bacto agar 15 g

SOB- medium:

Bacto tryptone 2%, Yeast Extract 0.5%, NaCl 0.05%, KCl 2.5 mM, $MgCl_2$ 10 mM

SOC- medium:

SOB medium, 20 mM Glucose

Transformation is the genetic alteration of a cell resulting from the uptake and expression of foreign genetic material. Bacteria are made artificially competent by passively making cells permeable for foreign DNA using methods that do not normally occur in nature. The plasmid being used for transformation should contain an origin of replication for independent replication and a selectivity marker that differentiates transformed cells from non transformed cells. Max efficiency DH5α competent cells from Invitrogen were thawed completely on ice and gently mixed. 1 ng to 10 ng plasmid DNA was pipetted to 100 µl of competent cells. These cells were then incubated on ice for 30 min followed by a heat shock treatment at 42°C in a water bath for 45 sec. The micro-centrifuge tubes were then placed on ice for 2 min and 900 µl of SOC medium was added. The bacteria were allowed to grow with shaking at 150 rpm for one hour at 37°C after which they were spread onto LB agar plates with the respective selection marker expressed from the transformed plasmid and grown overnight. Individual colonies were inoculated for culturing bacteria for plasmid isolation or protein purification.

2.2.2 Growth of bacteria: plasmid isolation

Bacterial colonies were streaked on LB agar plates. Individual colonies were inoculated in 5 ml culture and were used as preculture. This was inoculated at 1:100 dilutions in larger culture volumes. The strain of bacteria used for plasmid isolation was DH5α and the plasmids were isolated from this culture according to the manufacturer's (Qiagen) protocols using Qiagen Mini prep or Maxi prep kits.

2.2.3 Storage of bacteria

The exponential (or log) phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. This is measured by reading the optical density (OD) of the cultures at a wavelength of 600 nm. For storage of transformed bacteria, pelleted fresh log phase cultures were mixed with sterile glycerol (final concentration 20%) and stored at –80°C.

2.2.4 Agarose gel electrophoresis

TAE-buffer:

50 mM Tris pH 7.4, 20 mM Na-Acetate, 2 mM EDTA

TBE-buffer:

40x 1.8 M Tris-HCl, pH 8,5, 1,8 M Borate, 0.04 M ETDA

DNA Loading Buffer and dye:

1x TAE-buffer, 0.01% Bromphenol blue, 40% Glycerine

Agarose gel electrophoresis is a powerful analytical method for the separation of biomolecules. DNA is composed of a negatively charged phosphate group. The gel-electrophoresis apparatus uses a positively and a negatively charged pole generated by electrical currents. In the presence of charge, larger molecules move slower than smaller molecules, therefore the DNA molecules separate based on their respective sizes. The DNA is loaded in wells present on the negatively charged pole and allowed to pass through the gel towards the positive pole. DNA samples were prepared with 6X agarose gel loading buffer. These were loaded on 1% agarose gels in TAE or TBE buffer with 5% ethidium

bromide. The gels were run at 120 V until the dye reached half the length of the gel. The gel was then observed on a UV trans-illuminator and photographed. The DNA samples were loaded along with a DNA marker to determine the size of the bands observed.

2.2.5 Restriction enzyme digestion of the pET -huLa -WT and pET -huLa -MutΔ2 plasmids

Restriction enzymes cut DNA at a specific target nucleotide sequence thereby generating a reproducible set of fragments. The pET -huLa -WT and pET -huLa -MutΔ2 plasmids were digested with EcoR I in order to linearise the plasmids. 0.1 units of the enzyme EcoR I was added to 100 ng of DNA along with 3 µl of the reaction buffer supplied from New England Biolabs and the volume was made up to 20 µl. The reaction was carried out O/N at 37°C. This reaction was loaded on an agarose gel to observe the lengths of the fragments generated.

2.2.6 DNA concentration

The concentration of the DNA samples was quantified using GeneQuant II RNA/DNA calculator (Pharmacia Biotech Europe GmbH) in 10 µl Hellma quartz suprasil glass cuvettes (10 mm). Milli Q water or the buffer in which the respective DNA was eluted was used for calibration of the photometer. 5 µl were added to the cuvette and the concentration measured by UV absorption at 260 nm. A260 and A280 are the optical spectrometer measurement of absorbance at the wavelengths of 260 nm and 280 nm respectively. A260 is frequently used to measure DNA/RNA concentration and A280 is used to measure protein concentration. A ratio of A260/A280 > 1.8 suggests little protein contamination in a DNA /RNA sample. When the DNA samples were too concentrated, they were further diluted 1:5 or 1:10 times before they were measured and then the concentration was calculated.

DNA concentrations were calculated using the equation:

Concentration = A260 nm x dilution factor x 50 mg/ml (1 A260 nm unit of ds DNA = 50 mg/ml)

Determining the OD ratio (A280 nm/A260 nm) approximated DNA purity. A ratio value of 1.8 was considered to indicate DNA purity, with lower and higher values indicating RNA- and protein contamination, respectively accordingly (276).

2.2.7 WT La/Mut La protein production *in vivo* from recombinant bacteria

Lysis Buffer:

50 mM NaH_2PO_4 pH 8.0, 10 mM imidazole, 300 mM NaCl, 4% protease -inhibitor Complete

Wash Buffer:

50 mM NaH_2PO_4 pH 8.0, 42.5 mM imidazole, 1 M NaCl, 0.11% Triton-X100, 1% protease -inhibitor Complete

Elution Buffer:

50 mM NaH_2PO_4 pH 8.0, 300 mM imidazole, 300 mM NaCl, 1% protease -inhibitor Complete

Dialysis Buffer:

10 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 0.5 mM EDTA, 5% Glycerine

BL21 bacterial colonies transformed with the respective plasmids (pET -huLa -WT and pET -huLa -Mut Δ2) were streaked on LB Agar plates and from these individual colonies were inoculated in 5 ml culture and were used as preculture. This was inoculated at 1:100 dilutions in larger culture volumes. The strain of bacteria used for plasmid isolation was BL21. Absorbance was measured at 600 nm (A600).

The pET hLa plasmids (pET -huLa -WT and pET -huLa -Mut Δ2) used have a His tag that allows for the purification of the proteins expressed in *E.coli* using Qiagen's Ni-NTA (Nickel-nitrilotriacetic acid) metal affinity chromatography matrices for biomolecules having 6 consecutive histidine (6xHis) residues as an affinity tag. The 6xHis tag facilitates binding to the Ni-NTA agarose. Imidazole at higher concentrations facilitates the elution of the His tagged protein. The lysis buffer contains 10 mM imidazole to minimise binding of untagged contaminating proteins and increase purity with fewer wash steps. The wash buffer also contains imidazole at lower concentrations in order to stringently wash out any endogenous proteins with histidine residues.

IPTG was added to a final concentration of 1 mM in culture when the A600 (at 37°C with shaking of 200 rpm overnight) reached 0.5. IPTG is a non fermentable analog of lactose and

induces expression of genes under the control of a promoter based on the lac operon in bacteria that utilise lactose and can inactivate the lac repressor. After this the culture was shaken for 4-5 hours at 37°C, during which time the desired protein was expressed. The bacterial culture was then centrifuged and the pellet was resuspended in chilled lysis buffer (50 mM NaH_2PO_4, 10 mM imidazole and 300 mM NaCl) with 4% protease inhibitor and sonicated 3 times for 10 sec each to lyse the bacterial cell membranes. The supernatant of this lysate was then maintained on ice for all further steps of Ni-NTA agarose gel based batch purification or spin column purification. The wash buffer used contained 50 mM NaH_2PO_4, 42.5 mM imidazole, 1 mM NaCl and 0.11% Triton X 100. The column or agarose (in the batch process) was washed 2 times after which elution buffer (50 mM NaH_2PO_4, 300 mM imidazole and 1 mM NaCl) was used to elute the protein bound to the column. The total volumes of the buffers used for each method are as listed below:

	Spin columns	Batch process
Volume of culture used	5 ml	500 ml
Volume of agarose used	2 columns	2 ml
Volume of lysis buffer (10X)	0.5 ml	50 ml
Volume of wash buffer	600 µl	8 ml
Volume of elution buffer	300 µl	4 ml

This eluate was then dialyzed overnight at 4°C and concentrated with Amicon concentrators from Millipore as per the manufacturer's instructions. Protein concentration estimation was made by Biorad protein assay method (as described in section 2.2.8) and evaluated after Western blot analysis.

2.2.8 Protein concentration estimation

The Bradford method was used to measure protein concentration. This method is used to measure solubilised protein. Bovine Serum Albumin (BSA) was used as standard and 5X Bradford reagent from Bio-Rad was used for protein estimation. This reagent is acidic, containing Coomassie brilliant blue dye that changes colour corresponding to the concentration of the protein present and this colour change can be read on a spectrophotometer or a microplate reader at 595 nm. This reagent was prepared by diluting

it to 1X before it was added to samples. All steps were done according to the manufacturers instructions. Standards were made from 1 to 5 μg/ml BSA protein as 5 dilutions. Samples were diluted further if needed (when the blue colour arising after the sample was added to the Bradford reagents, was visibly more than the 5 μg/ml BSA standard). This was then incubated for 30 min at RT and 200 μl were transferred into a well of a 96 well microtiter plate. Using a microtiter plate reader (SLT spectra III plate reader from SLT Lab Instruments), the protein concentration was measured at 595 nm and the Magellan 3 software was used to calculate the concentrations. The protein concentrations were calculated using the BSA standard curve.

2.2.9 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE protein loading dye 4X:

0.4 M Tris-HCl pH 6.8, 10 mM EDTA, 0.1 M β -mercapto ethanol, 20% Glycerine, 8% SDS, 0.06 g Bromophenol blue for 10 ml dye

Running Gel buffer:

250 mM Tris (pH 8.8), 19.2 mM Glycine, 0.35 mM SDS

Spacer Gel Buffer:

0.5 M Tris, 0.4% SDS, pH adjusted to 6.76 with conc. HCl (37%)

Running gel buffer:

1.5 M Tris (pH 6.8), 0.4% SDS, pH adjusted to 8.8 with conc. HCl (37%)

The SDS PAGE method separates proteins according to their molecular weights (277). The electrophoretic mobility of proteins in gels will depend on both the size as well as the charge-to-mass ratio. Therefore, to separate proteins according to size it is necessary to give them to same charge-to-mass ratio. This can be accomplished by denaturing the protein in SDS (sodium dodecyl sulfate, shown below). The SDS binds to proteins in a uniform manner giving all proteins the same charge-to-mass ratio. SDS also denatures the proteins and β-mercaptoethanol cleaves disulphide linkages.

The protein samples to be analyzed were prepared with protein loading dye with 0.5% β-mercaptoethanol and were heated at 95°C for 3 minutes. The samples and appropriate markers were loaded onto 10% or 12.5% SDS PAGE gels and run at 125 V in the spacer gel

and 180 V in the running gels. The gels with radioactive protein were run until before the dye phase ran out of the gel and the non radioactive protein gels were run until the fragment of interest was well separated on the gel.

If the gels had to be blotted as was the case for Western blot analysis, they were done so at this stage (see section 2.2.10). Depending on the amount of protein in the gel, it was either stained using the Coomassie blue staining procedure or Silver staining. The gels were dried in moist thin foils overnight at room temperature.

2.2.10 Immunodetection (Western Blot)

TBS (10X):

30 g Tris base, 80 g NaCl, 2 g KCl, H_2O to 1 L, pH 7.4 with 1 M HCl and autoclave

TBST Buffer:

1X TBS plus 0.05% (v/v), Tween 20

Western-Blot Transfer buffer:

48 mM Tris-HCl , 139 mM Glycine, 20% Methanol, 0.05% SDS

Transfer Buffer:

48 mM Tris, 20% Methanol, 39 mM Glycine, 0.05% SDS

Western blotting was done on the semi-dry blotting apparatus at 180 V for 20 minutes. The nitrocellulose membrane (Hybond-ECL) from Amersham Biosciences was used for blotting and was prepared according to the manufacturers instructions. The gel was placed on the membrane and these lay in between a sandwich of 8 Whatman papers (4 on each side) soaked in transfer buffer. After blotting, the transfer of proteins on the membrane was judged either using Amido Black or Ponceau-S staining (reversible) as described below. The gels were stained using Coomassie staining or silver staining as described in sections 3.2.11.

Amido Black staining solution:

2X concentrate from Sigma

Amido Black destaining solution:

Aqueous solution containing 25% isopropanol, 10% Acetic Acid

Ponceau-S Staining solution:

0.5% (w/v) Ponceau-S, 1% Acetic Acid

The blotted membranes were stained with Amido black or with Ponceau-S (reversible staining) in order to control the amount of protein transferred. This was done according to the manufacturer's instructions. Usually Ponceau-S was used when the membranes were further used for Western blot analysis. This step confirmed blotting of the proteins from the gel to the membranes.

2.2.11 Antibody binding and Chemiluminescence reactions:

The nitrocellulose membrane obtained after Western blotting was blocked with 5% milk in TBST for an hour and then the blot was incubated in primary antibody (anti-La 4B6) at a dilution of 1:500 in blocking buffer overnight at 4°C. Washes were carried out twice with TBST before the secondary antibody HRP-conjugate goat anti-mouse was added in blocking buffer at a dilution of 1:5000. Similar washes were carried out. The membrane was detected using ECL kit from Amersham. The Enhanced Chemiluminescence (ECL) reagent from Amersham Biosciences is a light emitting non-radioactive method for detection of immobilised specific antigens, directly or indirectly with Horseradish Peroxidase (HRP) labelled antibodies. HRP catalyzes the oxidation of luminol in alkaline conditions and this is the principle used in ECL where this reaction is done in the presence of chemical enhancers such as phenols resulting in increasing both the intensity as well as the time of light emission. This light produced at a wavelength of 428 nm is detected by a short exposure to blue light sensitive autoradiography film. Equal amounts of solution 1 and solution 2 were added to the membrane, enough to cover it completely and incubation times varied between a few seconds and 10 min depending on the amount of protein on the membrane. These membranes were then exposed to Kodak Biomax XAR films, which were developed using the Agfa Curix 60 machine.

2.2.12 Staining of protein gels

2.2.12.1 Staining of gels with Coomassie blue staining

Coomassie staining solution:

0.2% Coomassie R-250, 20% Methanol, 5% glacial acetic acid, water up to 2.25 L and filtered.

Coomassie destaining solution:

5% Methanol, 7.5% Acetic Acid

Proteins on the gels were stained using coomassie blue staining. Coomassie stain was added to the gels, enough to cover them completely and staining was allowed for 20 minutes, after which the solution was decanted and destaining solution was added or the gels were allowed to destain in water overnight.

2.2.12.2 Silver Staining of protein gels

Silver Staining: Fixer:

45% (v/v) Methanol and 10% (v/v) glacial acetic acid

Silver Staining: Farmer's reducer:

0.03 M K_3 $Fe(CN)_6$ and 0.032 M $Na_2S_2O_3.5H_2O$

Silver Staining: Developer:

2.5% (w/v) Na_2CO_3 and 0.09% (v/v) Formaldehyde

Silver Staining: Stop solution:

10% glacial acetic acid in water

Silver staining was used as a more sensitive alternative to Coomassie staining of polyacrylamide gels. The protocol is a modified protocol (278). The gel was fixed with fixing solution for half an hour and then farmer's reagent was added to the gels for 1 minute. The gel was then washed with water until the yellow colour was completely removed from the gels and then the gels were incubated for 15 min in Silver nitrate solution. They were then developed with 2.5% Na_2CO_3 and 0.09% formaldehyde until the protein bands could be observed. The reaction was stopped with stop solution containing 10% acetic acid.

2.2.13 WT La/Mut La protein production *in vitro*

Single tube *in vitro* protein production was made using the TNT Quick coupled transcription & translation system with T7 promoter from Promega. The TNT Quick coupled transcription & translation system combines RNA Polymerase, nucleotides, salts, and RNasin ribonuclease inhibitor to form a single quick master mix. 1 µg of DNA was added to the TNT master mix. In addition to 2 µl of plasmid DNA (0.5 µg/µl) to be transcribed, 5 µl of S^{35} methionine from Amersham was added the reaction was filled up to 100 µl using nuclease free water. The reaction was allowed to proceed for 60 min at 30°C. These reaction mixes were either used immediately or frozen at -20°C until they were used or evaluated. The control used was the reaction mixture without DNA, as recommended according to the manufacturer's protocol. The results of translation were analyzed by SDS PAGE and Western blot analysis.

2.2.14 Detection of radioactive protein on gels

The dried gels were exposed to X-ray film directly or else onto Phosphor Imager screens. The X ray films were developed in an Agfa Curix 60 machine. The phosphor imager screens were subjected a FUJI FILM BAS 2500 reader and analysed by AIDA software. The exposure of gels to films lasted from a few seconds to a few minutes, the exposure to phosphor imager screens was normally done overnight. The used phosphor imager screens were erased using the Ray test eraser and the types of screens used were usually M type.

2.2.15 Peptide Synthesis on cellulose membrane library using the SPOT synthesis technique

Spot synthesis was the method used to synthesise peptides on cellulose membranes and these libraries were screened using mutant and wildtype La in order to find possible peptide binding partners. The syntheses for these libraries were carried out on the Abimed ASP222 Automated SPOT Robot (95). An amino-PEG cellulose membrane was used having a loading of 0.4 mMol /cm^2. 425 spots can be synthesised on a 8x12 cm membrane. Arrays of spots providing suitable anchor functions for peptide assembly on cellulose membranes are most easily generated by a two-step procedure. The first step involves preparation of the amino paper through esterification of an αN-Fmoc-protected amino acid to make available

the hydroxyl functions on the cellulose fibres of the whole sheet, followed by Fmoc cleavage. For this, the cellulose sheet is soaked in a solution containing 0.2 M Fmoc-β-alanine, 0.3 M DIC and 0.3 M NMI in dry NMP for 1-4 hours. The cellulose sheet is first washed in the reaction trough twice and left overnight with acetylation mix (capping mix). The sheet is then washed three times using 20 ml DMF, then incubated in 20% piperidine/DMF for 20 min to cleave the Fmoc protecting groups and then again in 20 ml DMF four times, with 20 ml alcohol three times and then dried in a desiccator overnight. Finally it is stored at -20°C. For long term storage, the Fmoc protecting groups should be left. The treatment with 20% piperidine/DMF is then carried out before the second step.

The second step involves spot-wise coupling of a suitable anchor compound (such as βAla-βAla-anchor) or another Fmoc amino acid which generates the array of spot reactors. All residual amino functions between spots are blocked by acetylation (2% acetic anhydride in DMF). 0.2 M of the second anchor compound (such as Fmoc-βAla-OH), 0.3 M HOBt and 0.3 M DIC in NMP was prepared (for this step only 0.1 µl was spotted). BPB was added and left for 30 min. Active ester between the HOBt and the βAla is formed to attack the free amino functions on the cellulose paper. Aliquots of this solution were spotted to all positions of the array and this reaction was allowed to be carried out for 30 min. Washes were done with 20 ml of acetylation mix for 30 sec and then 2 min and finally the sheet was left overnight in the acetylation mix (cap all amino functions between every spot, only till the second βAla step). This was again followed by washes with 20 ml DMF three times, incubation for 5 min with 20 ml of 20% piperidine/DMF. This was followed by further washes with DMF four times and incubation with 20 ml BPB (1% BPB v/v from 10 mg/ml stock in DMF) until the spots stain light blue. This is followed by a 20 ml alcohol wash (done twice). The sheet is dried with cold air from a hair dryer and can be used immediately or can be stored in a sealed plastic bag at -20°C.

The amino acids for building up the peptide chain are dissolved at 0.2 M ratio in NMP containing 0.3M HOBt and are stored at -70°C (this is the amino acid stock solution). Activation of the amino acids was done using 4 µl DIC (0.4 M DIC) per 100 µl amino acid stock solution at room temperature for 30 min. Vials containing activated amino acids were placed in a synthesiser rack and a spotting protocol was initiated. 0.2 µl of the amino acid solution was dispensed up to 3 times on a single spot for every spotting step. Capping of the

amino acids was done using 2% acetic anhydride in DMF for 30 sec and then 10 min in fresh acetylation mix. This was followed by 3x10 min washes in 20 ml DMF. The next step in the synthesis was deprotection of the amino functions by the cleavage of Fmoc by 20% piperidine in DMF for 5 minutes followed by 4x10 min washes with DMF. At this stage, a bromophenol blue (BPB) reaction was done in order to detect free amino groups. This was followed by washes with ethanol 3 times. The sheet was then dried with cold air from a hair dryer after every step.

The next elongation cycle of peptide synthesis, which consisted of activation of amino acids, spotting, capping, Fmoc deprotection, washing and drying were repeated in order to couple the next amino acid.

In the last step of SPOT technique based peptide synthesis, deprotection of the Fmoc group was done without prior capping, followed by the BPB reaction. This served as an indicator of synthesis as well as an indicator of the next capping step (2% acetylation mix, 2x15min) because the amino functions were blocked until the blue colour from the BPB reaction disappeared. This is followed by washing steps, 3x10 min DMF, 3x10 min ethanol and then the membranes are dried. After the last cycle, the side chain deprotection was done for two times one hour each using 50% TFA, 3% TIBS, 45% DCM, 2% H_2O (20 ml of deprotection mix) with gentle agitation. After deprotection, the membrane was washed for 4x10 min with DCM, 3x10 min with DMF, 3x10 min with ethanol, 3x10 min with 1M Acetic Acid (in water) and finally for 3x10 min with ethanol. Membranes were air-dried and stored at -20°C. These membranes could now be used for biological assays such as ligand/antibody binding.

2.2.16 Binding assays on cellulose membranes with WT and Mut La proteins

2.2.16.1 Radioactive WT and Mut La proteins used as probes (made using the pET -huLa -WT and pET -huLa -MutΔ2 plasmids)

La protein binding buffer:

10 mM Tris HCl pH 7.4, 150 mM NaCl, 3 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.5% NP-40

Stripping buffer:

0.06 M Tris HCl pH 6.8, 0.1 mM Beta mercapto ethanol , 2% SDS

The cellulose membranes were washed 2 times in 70% Ethanol and 2 times with the La protein binding buffer. They were then blocked overnight in 5% skim milk. 100 µl radioactive Mut Δ2 La probe made using the TNT kit was added to these membranes and binding was allowed for 4 h. This membrane was then washed four times for 10 minutes each in La protein binding buffer and then exposed to a phosphor imager screen overnight. The screen was read in the phosphor imager and signals were analysed using the AIDA software. Later the membrane was stripped using stripping buffer and the binding assay was repeated for WT hLa probe from the TNT kits. Based on the binding signals observed, further membranes were synthesised and binding assays were done with them in the same manner.

2.2.16.2 Non radioactive WT and Mut La proteins used as probes (made using the pET -huLa -WT and pET -huLa –Mut Δ2 plasmids)

La protein binding buffer:

10 mM Tris HCL pH 7.4, 150 mM NaCl, 3 mM $MgCl_2$, 0.5 mM EDTA, 1mM DTT, 0.5% NP-40

Stripping buffer:

0.06 M Tris HCl pH 6.8, 0.1 mM Beta mercapto ethanol , 2% SDS

The cellulose membranes were treated as above and blocked in a similar way. Non radioactive recombinant Mut Δ2 La protein made in *E. coli BL21* was added to these membranes and binding was allowed for 4 h in La protein binding buffer. The membrane was then washed with La protein binding buffer four times for 10 minutes each and after incubation with Amersham's ECL kit were exposed to Fuji X ray film. Later the membrane was stripped using the stripping buffer and the binding assay was repeated in the same way for recombinant wild type La protein.

2.2.17 Peptide synthesis on polypropylene foils

The amino functions necessary for peptide synthesis on the foils were produced by derivatisation of a native polypropylene foil of hydroxyl functions. On these amino functions, were carried out the peptide SPOT synthesis as on the cellulose membranes (as

described in sections 2.2.15) with minor differences. Briefly, the amino acids to be used for spotting were dissolved in HOBt containing DMF in the case of PP foils (instead of NMP as in the case of cellulose sheets) and are stored at -20°C. Activation of the amino acids was done using DIC at room temperature for 30 min. BPB was not added to the amino acid spotting solution. Spotting was done manually per each patch. The spotting volume was between 0.5 µl and 1 µl per patch. All the capping steps are done using acetic anhydride for 1x30 sec and 1x2 min and finally for 1x10 min (unlike the first capping step in cellulose sheets which is overnight). This is followed by Fmoc deprotection of the amino acids and continuation with the next cycle of peptide synthesis (which consisted of repeating the previous steps). All the washing steps are reduced to 3 min for each step (unlike 10 minutes in the cellulose sheets). In the last step of SPOT technique based peptide synthesis, deprotection of the Fmoc group was done without prior capping (same as cellulose) and using the side chain deprotection mix which consisted of 82% TFA, 3% TIBS, 10% DCM and 5% water. The washing steps after side chain deprotection have the same times as on cellulose membranes. The patches are dried in air and can then be used.

Apart from this different chemistries for the cell based assays were used. The βAla support was used for the peptide synthesis on foils.

Derivatisation of polypropylene foils:

A commercial available polypropylene foil of 0.27 mm thickness has been derivatised to create different functional groups and surfaces in order to perform cell based assays. The dimensions of the hydrophilic patches used for the assays were between 2x2 and 3x3 mm^2.

Starting with the native polypropylene foil a mask, plotted with paint was applied to the foils before they were subjected to ozone treatment. At the non-painted areas this created a number of reactive species such as peroxides and hydroxyl functions and made this so called patches more hydrophilic than the with paint covered areas which are left hydrophobic. These hydrophobic areas are building the borders between the patches.

This foil with hydrophilic patches and hydrophobic barriers was the starting material for all following derivatisations and reactions. The reactions were done on the hydrophilic patches only, the hydrophobic barriers did not undergo the reactions. For the order of the reactions

to create the different surfaces see table 2-1. The operations carried out for derivatisation are specified below.

Ozonolysis:

A mask was plotted with paint onto the polypropylene foil surface. Ozone treatment was done for 4 to 8 hours. The mask was washed off with 3x3 min ethanol.

Reduction with boran dimethyl sulphide complex:

The foil was treated overnight with a 1 M solution of boran dimethyl sulphide complex in methyl tert.-butyl ether (MTBE). After washing with 1x for 5 min with MTBE the foil was incubated with a 1:1:1 (v/v/v) solution of 3 N NaOH, water and 30% H_2O_2 for 1 hour. This was followed by washing steps, each for 5 min with 1x1 N NaOH, 1x1 N HCl, 1x water, 2x DMF and 2x DCM and drying.

Polymerization:

For the polymerization a monomer or a monomer mixture were solved as a 7% solution in DMSO containing 0.5 mM copper-(II) acetate as catalyst to initiate the radical polymerization. To start the reaction the foil were placed in a closed reaction vessel, the polymerization solution were added and a nitrogen stream were applied in to the polymerization solution to remove all oxygen out of the solution and the vessel which otherwise would avoid the radical polymerization. Then the reaction vessel were placed at 80°C in an oven and left for 24 h. After removing the foil out of the polymerization solution it was washed with each 3x3 min with 1 N NaOH, 1 N HCl, water and ethanol. The foils were air dried.

Polymers used:

DMA	N,N-dimethyl acryl amide
APMAA	N-(3-aminopropyl) methacrylamide hydrochloride
ThMAA	N-[Tris(hydroxymethyl)-methyl]acrylamide

For the mixture of monomers the ratios were for ThMAA/DMA 1:3 and for APMAA/ DMA 1:2.

CDI activation:

To make the hydroxyl functions of ThMAA accessible for further reactions the foils were for 4 to 6 hours treated with a 0.5 M solution of carbonyldiimidazole (CDI) in DMF.

Amine reaction:

The CDI activated foils were placed in a trough and overnight treated with a 60% solution of the desired amine in DMF. Only for the treatment with glucosamine hydrochloride were solved as a 0.5M solution in water and 1N NaOH adjusted to pH 7.5. After washing with 3x3 min with DMF and 3x3 min with ethanol the foils were air-dried.

The amines used were:

DACH	trans-1,4-diaminocyclohexane
Jeffamin 130	O-(2-Aminopropyl)-O'-(2-methoxyethyl)polypropylene 130
Jeffamin 500	O-(2-Aminopropyl)-O'-(2-methoxyethyl)polypropylene 500
Glucosamine	D(+)-glucosamine hydrochloride

A foil, derivatised according to the point# 9 in the table 2-1 was used to perform the peptide synthesis according to section 2.2.15.

Coating with Poly Lysine:

The foils were treated overnight with a 1:20 dilution of a poly lysine solution (100 μg/μl) in 0.1 M PBS, pH 7.4. After washing with 3x3 min water and 3x3 min ethanol the foil were air-dried.

Per Iodine cleavage:

Sodium metaperiodate, $NaIO_4$, is 3.3 M solved in a 20% solution of acetic acid in water. With this solution the glucosamine foil was treated for 10min, followed by washing with 3x3 min with water and 3x3 min with ethanol and then air-dried.

	Functionalisation method	Functional groups	Ozonolysis	Reduction	Polymerization (Monomer)	CDI Activation	Amine	Coating/ Spotting
1.	Ozonolysis	Peroxides, Hydroxyl functions	x					
2.	Ozonolysis + Reduction with Boran dimethyl sulphide complex	Hydroxyl functions	x	x				
3.	Polymerization with DMA	Methyl groups	x		DMA			
4.	Polymerization with APMAA/DMA	Amino functions	x		APMAA/DMA			
5.	Polymerization with ThMAA/DMA	Hydroxyl functions	x		ThMAA/DMA			
6.	Polymerization with ThMAA/DMA + CDI activation	CDI preactivated surface	x		ThMAA/DMA	x		
7.	Polymerization with ThMAA/DMA + Diaminocyclohexane (DACH)	Amino functions	x		ThMAA/DMA	x	DACH	
8.	Polymerization with ThMAA/DMA + Jeffamine (130)	Amino functions	x		ThMAA/DMA		Jeffamine 130	
9.	Polymerization with ThMAA/DMA + Jeffamine (500)	Amino functions	x		ThMAA/DMA	x	Jeffamine 500	
10.	Polymerization with ThMAA/DMA + Jeffamine (500) + Poly Lysine coat	Poly –L - Lysine	x		ThMAA/DMA	x	Jeffamine 500	Poly-Lysine coating
11.	Polymerization with ThMAA/DMA + Jeffamine (500) + β Alanine spots	β Alanine	x		ThMAA/DMA	x	Jeffamine 500	ßAlanine Spots
12.	Polymerization with ThMAA/DMA + Jeffamine (500) +	Lysine	x		ThMAA/DMA	x	Jeffamine 500	Lysine Spots

	Functionalisation method	Functional groups	Ozonolysis	Reduction	Polymerization (Monomer)	CDI Activation	Amine	Coating/ Spotting
	Lysine spots							
13.	Polymerization with ThMAA/DMA + Glucosamine	Sugar molecule	x		ThMAA/DMA	x	Glucosamine	
14.	Polymerization with ThMAA/DMA + Glucosamine + per iodine cleavage	Aldehyde functions	x		ThMAA/DMA	x	Glucosamine + per iodine cleavage	

Table 2-1. The type of surface chemistry done on the foils and the respective functional groups obtained are listed.

2.2.18 DT-2 peptide synthesis on polypropylene foils with cleavable linker

Foils with amino functions and the Jeffamine 130 spacer were used for the DT-2 peptide synthesis (279). The coupling was of a carboxy-Frank-Linker DHCA (2-(1-t-Butyloxycarbonyl-4-methyl-imidazol-5-yl)-2-hydroxy-acetic acid dicyclohexylamine). The linker was used as a 0.2 M solution in DMF, 2 equivalents HOBt and 1.5 equivalents DIC were used for preactivation for 20 min. Spotting of the linker solution was done twice onto the foil, working up as after normal peptide synthesis cycle. On this linker-foil the DT-2 peptide was coupled, starting again with the esterification of histidine as first step. Peptide synthesis and also the side chain deprotection with TFA was then carried out like in section 2.2.15 and 2.2.17. These were then used for cell based assays. The DT-2 peptide was released into media by using a solution with neutral pH such as cell culture media. This was due to the characteristic of the linker which has the property to be cleaved in neutral pH.

2.2.19 Cell Based Assays on polypropylene foils with hydrophilic patches

The different cell lines were grown in their respective media (Table 2-2) and after counting on a haemocytometer the final concentration was adjusted to 500,000 cells/ml.

The cells were counted on a Neubauer chamber according to the following equation:

Cells/ml = (average count /square) x (dilution factor) x 10^4 [chamber conversion factor]

Cell line	Medium	Serum	Number of passages per week	Proportion inoculated for next passages	Method to de–adhere cells from the surface
L929	DME	FBS/NCS	1x	1:20	Scrapped
PtK2	MEM+	FBS	1x	1:10	Scrapped
A498	MEM+	FBS	1x	1:10	Scrapped
PC-3	F12K	FBS	1x	1:10	Scrapped
SKOV-3	Mc Coy	FBS	1x	1:10	Scrapped
A431	RPMI	FBS	1x	1:20	trypsinised
Hela	DMEM	FCS	1x	1:20	trypsinised
3Y1	DMEM	FCS	1x	1:10	trypsinised
HepG2	DMEM	FCS	1x	1:10	trypsinised
Huh7	DMEM	FCS	1x	1:10	trypsinised
A549	DMEM	FCS	1x	1:20	trypsinised

Table 2-2. Mammalian cell lines used and their respective cell culture requirements

2.2.20 Freezing of mammalian cells:

Cell Freezing Medium:

Cell culture media plus 10% DMSO

In order to preserve the cells used for the various experiments done here, they were grown on tissue culture plates and were trypsinised or scrapped and resuspended in cell culture medium. After this the cells were centrifuged at 1000 g for 3 min at 4°C. The cell pellet was resuspended in ice-cold cell freezing medium at a concentration of 0.5 x 10^7 cells/ml and then slowly brought from 4°C to liquid nitrogen. A frozen vial of cells was thawed after a certain number of passages for the each cell line.

2.2.21 Growth of cells on patches

The desired number of patches was selectively cut from the polypropylene foils (usually 6 to 8 depending on size of patch). This foil was then washed 2 times with 70% ethanol and 3 times with PBS (5 min for each washing step). 5 µl of media having 500,000 cells/ml were added per patch on all the patches on a foil. These were then put in 3.5 cm diameter culture plates and 3 such plates were put in a 10 cm petriplate that is filled with water to facilitate humidity in the plate and prevent the evaporation of media from the individual drops over time. The cells were incubated at 37°C with 5% CO_2 and grown over 2 days and then analysed and documented with photographs from the individual patches in order to study characteristics such as cell growth, adherence to the patch, clustering, monolayer forming ability and cell viability. The microscope used for this study was the Olympus SZX 12.

2.2.22 Monitoring parameters of normal cell growth using Trypan Blue

One of the simplest ways to monitor normal cell growth is using Trypan Blue. It is a biological stain/viable dye that stains dead cells blue and is excluded by cells having an intact cell membrane. Trypan Blue is a tetrasulfonated anionic dye and has widely been used as a vital stain since the early 20th century. This dye has a wide variety of other applications and can be used to stain collagen, amyloid, as a quenching agent to suppress auto-fluorescence in tissue section, in flow cytometry as a fluorescent tracker of cell populations and also has several uses in biomedicine. 1 µl of dye was added to each of the patches. After an incubation time of 3 min, the liquid was gently pipetted off the patch and PBS was added to observe the cells for their viability on patches. Cell growth was compared on patches at various steps of peptide synthesis, in order to standardise the kind of linker to be used as well as to observe the effects of surface chemistry of the patches on the cells.

2.2.23 Repeating cellular phenotypes on patches

2.2.23.1 Lamellipodia induction in 3Y1 cells grown on PLL

Solutions and Buffers:

Stock of Poly L Lysine (PLL)	10 mg/ml in water
PLL –A	150,000 to 300,000 Da Molecular weight

PLL –B	30,000 to 70, 000 Da Molecular weight
Working solution of PLL	100 μg/μl

The poly L lysine (PLL) stock of 2 mg/ml was diluted 1:20 to obtain a working stock of 100 μg/ml. 100 μl of this was used per coverslip and 500 μl per piece of PP foil. Drops of PLL were first pipetted onto parafilm and placed in a humid chamber. The coverslips or foils were then laid with the cell growing surface facing the parafilm and in this way were incubated with PLL for 30 min. They were then thoroughly washed with PBS by dipping into beakers containing PBS. These steps were done fast in order to remove uncoated excess lysine. The coverslips and foils were placed right side up (cell growing surface) and stored at room temperature. Prior to use they were UV sterilised for 10 min.

The PP foils that were treated with PLL in the above method were categorised as PLL coated (not covalently linked) foils where van der Waals forces are the binding force of PLL to the PP surfaces. In order to covalently link PLL to the surface of these foils, a solution of 0.1 M PBS, 5% of the PLL stock and 0.1% of tween 20 were poured onto a square petriplate and the PP foils were carefully placed on this with the patch side facing the surface of the petriplate. These were then placed on a rocking table overnight and were subsequently washed with PBS, 70% ethanol and then 100% ethanol for 5 min, 3 times each. The foils were then dried and sealed in plastic sheets. The foils used for growing cells were cut so that each foil would have 6-8 patches each where cells could be grown. These foils were designated as activated covalently linked foils.

3Y1 cells were grown in cell culture plates and prior to plating on the activated foils, were trypsinated, counted and further diluted to obtain 5×10^5 cells/ml. 5 μl of the suspension was pipetted onto each patch. As coverslip controls, 500 μl of these diluted cells were further diluted in 2 ml media per 6 cm plate having 3 coverslips at its bottom. Cell growth was allowed for 2 days (280).

The cells were fixed with 4% PFA/0.25% gluteraldehyde for 20 min at 37°C. They were then twice washed with PBS and the cell membranes perforated for antibodies using pre-warmed 4% PFA/0.1% Triton X 100 in PBS for 30 sec. The coverslips or membranes were then incubated on a drop of 30-50 μl of Alexa 488 Phalloidin for 45 min at RT in a humid chamber followed by washes in PBS. These were then mounted using Mowiol and n-propyl gallate

(npg) as anti-fade reagent on slides. These were stored at 4°C until photographed and analyzed using the Axiovert Microscope from Zeiss. IPLab spectrum was the software used for taking photographs and analyzing the pictures taken.

2.2.23.2 Inhibition of cytokinesis in PtK2 cells grown on foils with DT2 peptide synthesised

DT-2 peptide with the sequence YGRKKRRQRRRPPLRKKKKKH (Mol Wt. 2800) (281) was synthesised on the patches as described in section 2.2.18. These patches were washed with ethanol and PBS pH 6.5. 5 μl of PtK2 cells were added to each patch. These cells were allowed to grow on the patches for 2 days at 37°C. After this, the patches with cells were washed with PBS and fixed with 37% formaldehyde for 10 min. After 3 washes in PBS, the patches were incubated with 0.05% triton X-100 in PBS. This step accounts for the permeability of cells to antibodies.

Following 2 washes with PBS, the patches were incubated with primary anti-tubulin antibody at a dilution of 1:500 for one hour (dilutions were done in 10% NCS and PBS). Later they were incubated for an hour with the fluorescein conjugated secondary goat anti-mouse antibody at a dilution of 1:200. These patches were then incubated with DAPI (final concentration 1 μg/ml) for 5 minutes and then mounted in anti-fade on glass slides. These were then observed with a confocal microscope having the respective filters and double-labelled images were taken with the Axioviosion software.

A basic peptide, pep1 (highly basic sequence -Ac-KETWWETWWTEWSQPKKKRKVC(SH)-amide, was used as a negative control on patches. It was given externally to the patches to the cells at a concentration of 10 μg/ml. DT2 peptide was also externally added to the patches with cells at the same concentration as the positive control. All experiments consisted of coverslip controls with the same conditions.

2.2.24 Transfection into mammalian cells

Fugene 6 was the transfection reagent used to transfect mammalian cells with the respective plasmids. FuGENE® 6 transfection reagent, a proprietary blend of lipids and other components supplied in 80% ethanol, forms a complex with DNA and then transports it into

animal cells. Cells were plated to 70% confluency. Medium was changed 3 to 4 hours before transfection. The ratio of transfection reagent used to the μg of DNA was 3:1. This ratio was found to be optimum for transfection efficiencies between 8 to 18 percent. These transfected cells were grown for at least 48 hours before they were used in further experiments.

2.2.25 GFP La nuclear export signal (NES) construct

In the GFP-C1-La-WT plasmid, C1 indicates the pEFGP vector (clonetech). La was cloned via SacI and HindIII. La sequence between nt 1354 and 2641 (La nt 91-1378). The three oligonucleotides for cloning the NES were chosen based on the speed at which they move cargo out of the nucleus. (282)

NES of the Protein kinase inhibitor (PKI), judged as fast NES (5 ± 10min)

```
        XhoI                    EcoRV     XhoI
PKI-S: 5′-T CGA GCT CAA TTA GCC TTG AAA TTA GCA GGT CTT GAT ATC AAC TC-3′
PKI-AS:3′-   CGA GTT AAT CGG AAC TTT AAT CGT CCA GAA CTA TAG TTG AGA GCT5′
             L  A  L  K  L  A  G  L  D  I  N
```

NES of HIV-1 Rev medium type 1 (10 ± 20min)

```
       XhoI                            XhoI
REV-S: 5′-T CGA GCT CAA CTT CAG CTA CCA CCG CTT GAG AGA CTT ACT CTT TC-3′
REV-AS:3′-   CGA GTT GAA GTC GAT GGT GGC GAA CTC TCT GAA TGA GAA AGA GCT5′
             L  Q  L  P  P  L  E  R  L  T  L
```

NES of Adenovirus type 5 E1B-55K slow (30 ± 60min)

```
       XhoI                            XhoI
E1B-S: 5′-T CGA GCT CAA CTG TAT CCA GAA CTG AGA CGC ATT TTG ACA ATT TC-3′
E1B-AS:3′-   CGA GTT GAC ATA GGT CTT GAC TCT GCG TAA AAC TGT TAA AGA GCT5′
             L  Y  P  E  L  R  R  I  L  T  I
```

1 μl of the oligonucleotides at a concentration of 0.05 μM were annealed in annealing buffer at 95°C for 4 min. The vector was XhoI digested and purified by gel extraction and then dephosphorylated. The annealed oligos were phosphorylated and ligated with the dephosphorylated vector using the rapid DNA ligation kit. The ligated mixture was

transformed into competent cells. Mini preps were performed and plasmids isolated. All the cloning was done in the lab of Dr. T. Heise.

2.2.26 Induction of apoptosis in transfected cells

On apoptosis induction, it would be expected that the WT hLa protein which is predominantly in the nucleus should be translocated into the cytoplasm (223). Apoptosis induces cleavage of the La protein and the aim was to reproduce the cleavage of La on apoptosis induction using known apoptosis inducers such as UV or chemical compounds like epothilone.

2.2.26.1 Induction using UV

HeLa cells were transfected with the GFP La plasmid and were allowed to grow for 48 hours before they were exposed to 100 J of UV light. Therefore, all media was aspirated to allow for proper apoptosis induction of the cells by placing the culture dish in a Stratagene linker and irradiating with UV. Then fresh medium was added to the cells and they were observed at different time points for cellular translocation of the La protein from cell nucleus to the cell cytoplasm. The cells were fixed in 3.7% formaldehyde for 10 min and washed with PBS. Then the coverslips were mounted in Mowiol containing npg and observed using fluorescence microscopy. The controls were transfected cells without UV treatment.

2.2.26.2 Induction of apoptosis using chemicals

A549 (lung carcinoma cells) were transfected with the pEGFP WT La and pEGFP NES WT La plasmids and were grown for 48 hours before they were treated with epothilone which induces apoptosis at a concentration of 50 ng/ml (283). The cells were fixed with 3.7% formaldehyde for 10 min and washed with PBS. The coverslips were mounted in mowiol containing npg, observed by fluorescence microscopy and photographed. The controls were transfected cells without epothilone treatment.

2.2.27 Cygnet La construct

The pcDNA 3.1 (-) -Cygnet-2 vector was kindly provided by Dr. Dostmann (275), University of Vermont, U.S.A. This vector has a fluorophore on either end, the ECFP at the C terminal end and the EYFP at the N terminal end. The other plasmids used for this cloning were pEGFP-La WT and pQe9. The Cygnet vector was digested with EcoR I/Xho I to remove the ECFP-PKG-EYFP cassette. The products after the digestion were cygnet EcoR I/Xho I vector (about 5400 nt) and ECFP-PKG-EYFP cassette (about 3225 nt). The pQe9 was digested with EcoR I/Xho I resulting in fragments with 90 nt and 3350 nt. Ligation of the ECFP-PKG-EYFP cassette into the pQe9-EcoR I/Xho I digest resulted in a pQe9-ECFP-PKG-EYFP fragment. This was then digested with Sac I and Sph I enzymes and the PKG fragment was removed. The elution resulted in a pQe9-ECFP-SphI-SacI-EYFP fragment.

A La fragment was raised by PCR using oligonucleotides 54AS/53S containing restriction sites for SphI and SacI. The PCR amplified La fragment was cleaved with Sph I and Sac I, gel eluated and ligated into the pQe9-ECFP-Sph I-Sac I-EYFP, leading to a ECFP-La-EYFP fusion cDNA. This part was removed by EcoR I/XhoI digestion from pQe9-ECFP -La-EYFP and ligated with cygnet EcoR I/Xho I cleaved vector resulting in the pcDNA 3.1 (-)-La-Cygnet-2 vector. The La borders were then sequenced to confirm in-frame ligation. All the cloning was done in the lab of Dr. T. Heise.

2.2.27.1 Fluorescence activated cell sorting (FACS)

HeLa cells transfected with the Cygnet La plasmid were sorted in a Mo Flo cell sorter (Dako, formerly Cytomation). The sorting was done based on the YFP fluorophore. The cells were sorted twice to have about 90% or more positively transfected cells. The sorting could also be done using the ECFP fluorophore. However it was found that EYFP, which gave a much stronger signal, was easier to sort. The software used to obtain graphs and follow the entire sorting procedure was Summit (version 3.1). The sorted cells were then lysed as described in the next section.

2.2.27.2 Lysis of transfected cells

Mammalian Cell Lysis Buffer:

150 mM NaCl pH 7.4, 10 mM Tris HCl pH 7.4, 0.5% NP40, 1X protease inhibitor

The cells obtained after sorting were centrifuged at 1000 rpm for 3 min and resuspended in 2 times (the volume of the cells) as much mammalian lysis buffer, according to a published protocol (284; 284). These cells were lysed for 10 min at 4°C. Later this lysate was centrifuged at 9300 rpm (10,000 g) for 10 min and the supernatant was carefully transferred into another eppendorf tube. This was then stored at -20°C and used for all further experiments. According to this lysis procedure, the supernatant contains the cytoplasmic contents of the cells and the pellet contains the unlysed nuclear fractions.

2.2.28 FRET *in vivo* measurements

HeLa cells were grown in chambers having a coverslip bottom. Transfection of the cells was done on these chambers using Fugene 6 transfection reagent. The ratio of transfection reagent used to the μg of DNA was 3:1. The cells were excited with light of wavelength 458 nm and emission spectra were recorded from 467 to 628 nm. The lens used was Plan Neo Fluar 100X Oil with a NA of 1.3. Then a certain defined region in a transfected cell was selected for photobleaching. This area was bleached for up to 8 min with 514 nm laser light that excites and bleaches the acceptor (EYFP). Hence this method of photobleaching is called acceptor photobleaching. After this, emission spectra were again recorded for the defined region as well as for the whole cell. The detector used in this case was a Meta detector and the software used for analysis was LSM 510 version 3.2.SP2. The filters used for imaging were with a band pass (BP) of 475 nm to 525 nm for ECFP and 530 nm to 560 nm for EYFP.

2.2.29 FRET *in vitro* measurements

2.2.29.1 Lysis of transfected cells

HeLa cells were transfected with the cygnet La plasmid and allowed to express the protein for about 36 to 48 h before they were sorted. Sorting was done on the basis of the EYFP

fluorophore. The sorted cells were lysed in cell lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 0.5% NP-40 and 1% protease inhibitor (according to the protocol from E. Chan et al.). The lysate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was used for all further experiments with protease digestion or FRET measurements *in vitro*.

2.2.29.2 FRET measurement

Emission spectra were recorded in quartz suprasil cuvettes for 400 µl of lysed cell samples (at 1 µg/µl) at excitation 430 nm for ECFP in the Fluoromax 3 fluorometer. The emission spectra were read from 480 nm to 560 nm, which is the expected range for EYFP emission. The spectra were exported from Instrument Control Center software and plotted in EXCEL for further comparative analysis.

2.2.29.3 Protease digestion of cygnet La transfected Hela cell lysate

Protease digestion buffer:

200 mM Tris-HCl pH 6.9, 2 mM EDTA

Protease digestion of wildtype La was carried out with Proteinase K (at 0.6 µg/ml, 1.8 µg/ml & 5.4 µg/ml), Chymotrypsin (25 µg/ml and 50 µg/ml) and Elastase (5 µg/ml and 90 µg/ml) (284). 1.5 µg of protein was digested in each case for 1 min, 10 min and 30 min and the reaction in each case was stopped by adding sample loading buffer and heating the sample for 3 min at 90°C. In case of Cygnet La, 50 µg of total cell lysate (cygnet La transfected HeLa cells) were digested with the same concentrations of restriction enzymes. And for the FRET spectrum measurement, 500 µl of sorted cell lysate at a concentration of 1 µg/ml were digested and used to read spectra. 20 µl of the digested sample was stored at -20°C after stopping the reactions with an appropriate amount of loading buffer, until it was loaded on an SDS page, blotted (western blotting) and bound to primary anti-La antibody 4B6. ECL detection was carried out after binding with HRP conjugated Goat anti-mouse secondary antibody.

3 Results and Discussion

3.1 Cell based assays on a novel platform for chemical synthesis

3.1.1 Comparison of the growth of different mammalian cell lines in response to patch surface chemistry

The main aim of this study was to establish a platform where cell based assays could be carried out on the same polypropylene (PP) plastic surfaces (referred to as PP foils or patches) having small molecules synthesised on them. On standardization, these assays would serve as a platform to screen libraries of small molecules against cells transfected with the La protein in order to identify compounds or peptides that would activate a protease that can target the La protein. The patches used for the cell based assays are shown in figure 1-5.

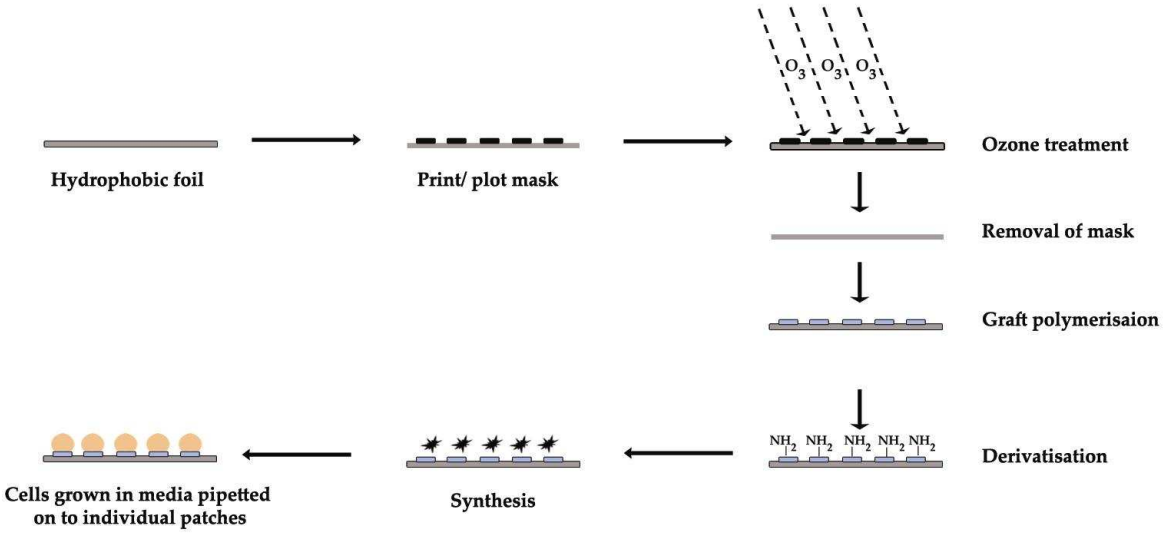

Figure 3-1. Steps involved in preparing the PP foils for synthesising small molecule compounds on the patches.
A mask was printed or plotted on the hydrophobic foil followed by ozone treatment. The mask was then removed and the foil is ready for graft polymerisation creating hydroxyl groups on the individual patches. Derivatisation creates amino groups on which further synthesis was carried out. These foils are UV sterilised before they can be used to grow cells.

Hydrophilic patches were created on PP foils by subjecting pre-masked foils to ozonolysis (see figure 3-1). Further chemical reactions were possible by polymerisation of different monomers (as described in section 2.2.17). Once the foils were ready, they were UV sterilised

for cell growth. Cell lines were tested for their suitability for growth on the patches. They were pipetted on to individual hydrophilic patches in their respective media requirements (as listed in section 2.2.18). A total of ten commonly used cell lines were tested on the patches.

3.1.2 Response of cells to the functional groups present on the patch surfaces

Cell lines were tested for their response to the surface chemistry used on the foils. Different functional groups were generated and cell behaviour towards these groups was observed. These behavioural assays were performed in order to be able to decide which surfaces and spacers could be used for the synthesis of compounds. From the ten cell lines tested on the different surface chemical modifications (see appendix 6.1), HepG2, Huh7 and Hela were of importance for this project as these most closely resembled human liver cells. At first, the reactions to simple surface chemical modifications (patches 1, 9, 10, 11, 13, & 15 as can be seen in the appendix 6.1) such as surfaces with hydroxyl and aldehyde functions were observed. Later a series of patches were synthesised with more complex surface chemical modifications including methyl and amino functions (patches V1 to V14), which could eventually be involved in synthesizing the different compounds on the patches. In each case a cell viability staining using trypan blue was done on the patches after cell growth in order to observe cell death on the individual patches. Based on cell growth, cell death and cell adherence, the suitability of the patch surfaces for cells was decided (see figure 3-2 for examples of how patches were graded based on cell behaviour).

Surface modifications with hydroxyl functions on ozonolysis gave the best results where cells grew as they did on coverslips. Amino functions were in comparison not as good, however they were rather important since they would form the basis for peptide synthesis on patches. Hence an effort was made to optimise cell growth on these. Examples of cell behaviour to patches can be seen in figures from appendix 6.1 and based on the gradation scheme used are summarised in table 6-1.

In this study Jeffamine has been used as a spacer. The JEFFAMINE® polyoxyalkyleneamines are a part of an ever-expanding family of polyether compound products. They contain primary amino groups attached to the terminus of a polyether backbone. They are thus "polyether amines" (poly(propylene glycol)bis 2-(aminopropyl) ether). The polyether backbone is based either on propylene oxide (PO), ethylene oxide (EO), or mixed EO/PO.

Newer products may contain other backbone segments and varied reactivity provided by hindering the primary amine or through secondary amine functionality. The basic JEFFAMINE® product family consists of monoamines, diamines, and triamines, which are available in a variety of molecular weights, ranging up to 5,000. The wide range of molecular weights, amine functionality, and oxide type and distribution provides flexibility in synthetic design of compounds made from JEFFAMINE® products.

The 130er and 500er Jeffamine (J130 and J500 respectively) have been used in this study as short and long spacers. Jeffamine spacers have been employed to tether antibodies to silicon wafer surfaces for the purpose of improving the orientation of antibody as well as reducing the steric hindrance (285). Other biological applications of Jeffamine include its use in reducing the calcification of bio-prostheses such as heart valve tissues in rat models (286; 287)

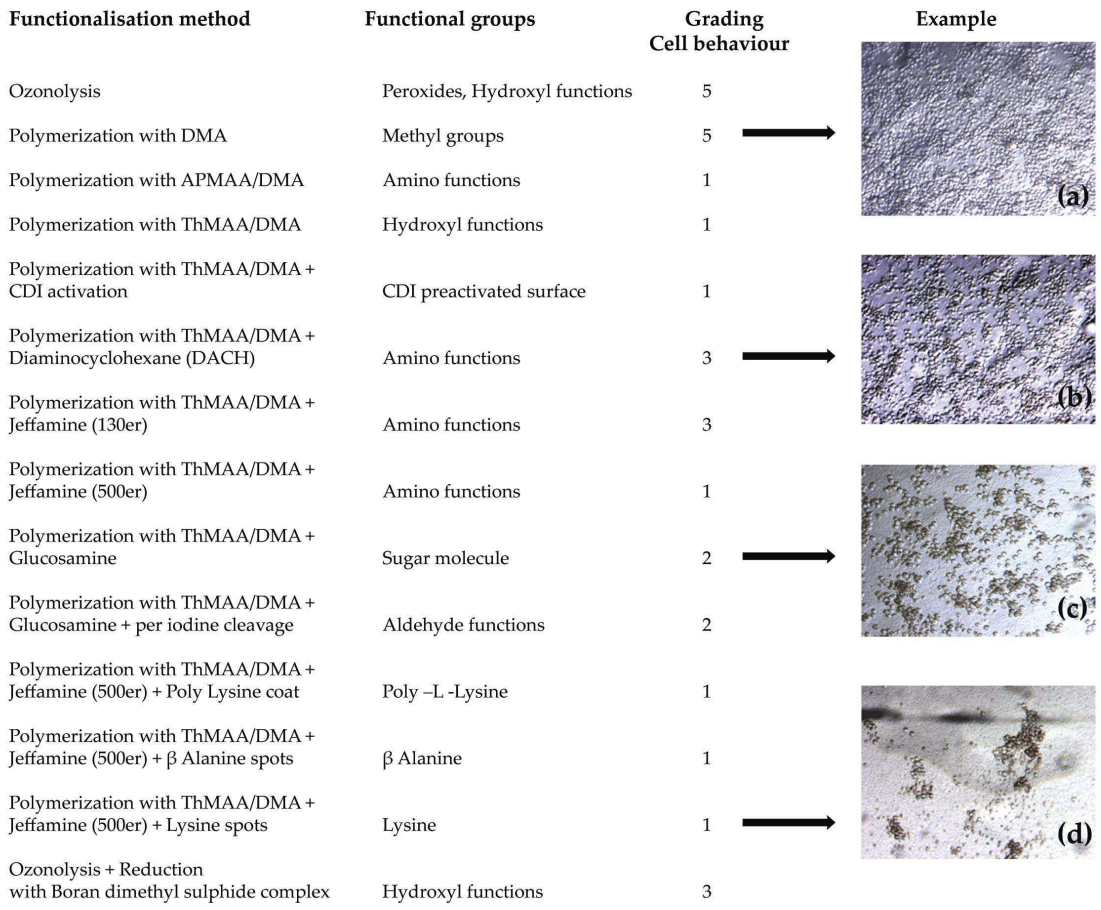

Figure 3-2. Examples of cell behaviour towards patch chemistry
The functionalisation methods used to obtain the different functional groups are listed. Also listed is the status conferred on each of the individual patches which depict whether the patch was considered acceptable or unfavourable based on a system of grading the growth of cells and viability on the

patches, Grade 1 (being the least acceptable growth) to Grade 5 (being excellent growth comparable to cell growth on glass coverslips). Since a quick screen was done visually based on which the suitability of the patches was determined, examples have been given of favourable patches for cell growth and less favourable ones. The examples given are of Hela cells grown on the respective patches, depicting conditions necessary for a patch to be considered acceptable. The cells depicted in each view are HeLa cells grown on 4 patches with different surface modifications. Panels (a) and (b) depict patches where cell growth was excellent (Grade 5) and good (Grade 3) respectively hence leading to these patches being classified as favourable for cell growth. Panels (c) and (d) depict cell growth on less favourable patches. These criteria determine which steps can be used during the chemical modification favourably for cell growth. The chemistry on the patches was intended to depict the various stages of small molecule synthesis. This would form the basis for the chemical modification steps which would ultimately be chosen to give rise to full length peptides. (Magnification: 90X, DMA: N, N-Dimethylacetamide, ThMAA: 2-N-(Acryloyloxyethyl)-N-methyl 1 thiophenecarboxamide).

It was observed that in general, cells preferred patches with amino groups on a shorter spacer as compared to the same groups on a longer spacer. This thus led to testing of patches with variable linker length (J130 and J500) and surface charge of the individual compounds, in this case completely synthesised peptides (section 3.1.3).

3.1.3 Response of cells to the property of an individual peptide and the length of spacers used

The responses of different cell lines to the charges of compounds synthesised on the surface as well as the effect of length of a linker were studied (figure 3-3 and appendix 6.2). The 130er and 500er Jeffamine (J130 and J500 respectively) have been used in this study as short and long spacers. Peptides that were highly basic (KKKK), acidic (DEDE), non polar hydrophobic (AVAV) and polar uncharged (GQGQ) were used to test the four extreme conditions of surface charge. It was observed that the cell lines were sensitive to surface charge as well as to length of the linkers used. The effect of charged surfaces was tested in 5 different cell lines (see appendix). Cells grown on patches with shorter spacer showed better growth and less cell death on trypan blue staining as compared to cells grown on patches with a long linker having the same peptide sequences as can be clearly observed in the figure 3-3. Also, in both cases, the patches with basic peptides (KKKK) showed better results as compared to the patches with non polar hydrophobic (AVAV). The acidic (DEDE) peptide and the polar uncharged peptides (GQGQ) resulted in a lot of cell death upon trypan blue staining. These are overall generalizations since the individual cell lines showed some variations. The length of the spacer or the charge of the peptides did not seem to affect the

HepG2 cells and PtK2 cells. However, the effect of the length of the spacer was profound in HeLa cells and Huh7 cells (appendix section 6.2).

This thus led to the conclusion that the type of surface chemistry on the patches influences the behaviour of the cells. Also, this varied from cell line to cell line. As with all high throughput techniques, the use of these patches would need some optimization especially for more sensitive cell lines as compared to the more robust ones. Also, it is important to note that in this case the tests have been made with peptides that were highly charged whereby the extreme results observed, can thus be justified. The results obtained from the experiments in this section enabled the further testing of the patches for full length peptides.

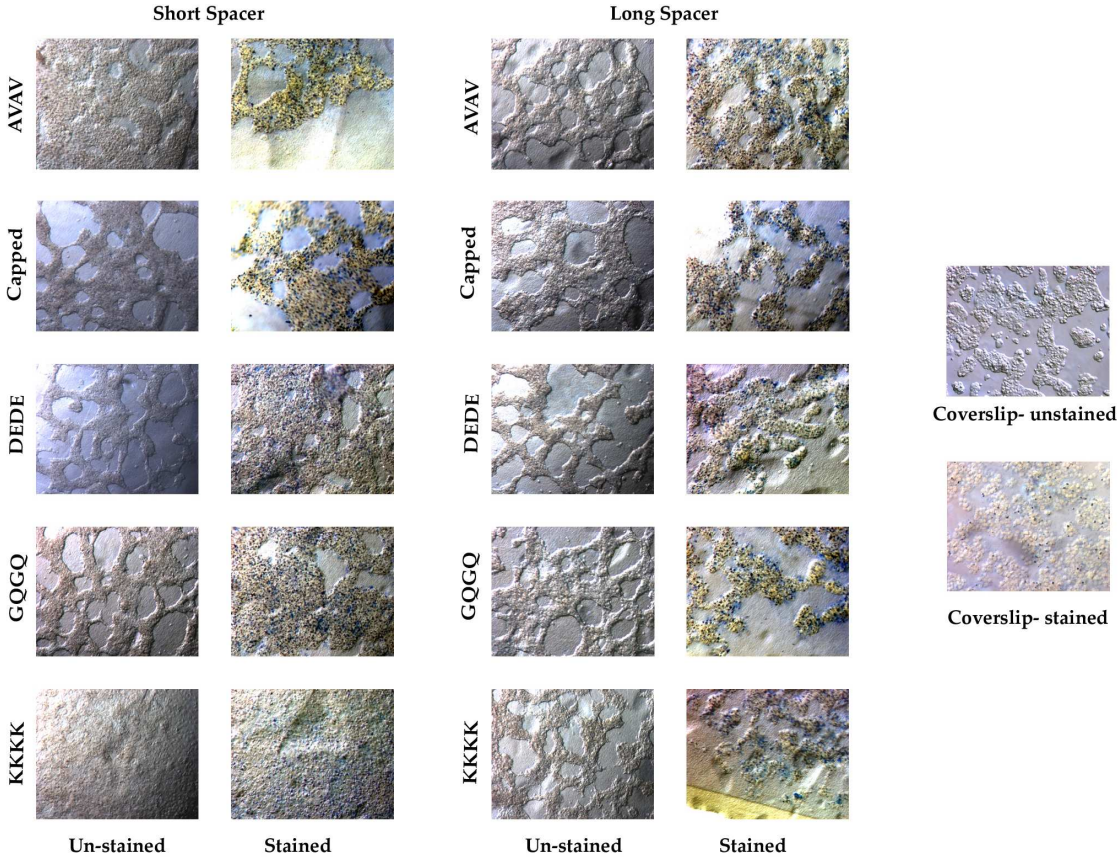

Figure 3-3. Comparing cells grown on PP foils synthesised with highly charged peptides
Behaviour of HepG2 cells towards the surface charge of patches as well as the effect of the length of these linkers used to link these peptides to the surface of the PP foils can be observed. (Magnification: 90X, AVAV: non polar hydrophobic, DEDE: acidic, GQGQ: polar uncharged, KKKK: basic, PEG: poly ethylene glycol

3.2 Reproduction of morphological phenotypes on polypropylene foils

Lamellipodia induction observed in 3Y1 cells plated on PLL coated patches

In order to check for the reproduction of morphological phenotypes on the PP foils, it was essential to check the foils for the duplication of a simple cellular phenotype such as cell shape. The shape of a cell in a differentiated tissue is dependent on its interactions with its neighbours and the extracellular matrix. Acquisition of cell form during development requires cytoskeletal reorganisation which is closely coordinated with the establishment of junctional structures that couple the cytoskeleton to extracellular ligands through transmembrane interactions. The actin cytoskeleton plays a major role in shape determination but its own reorganisation is subject to modulation by extracellular interactions and by microtubule-dependent activities. It is an integrated array of interconnected filament-assemblies, each with a defined architectural organization.

The most-cited assemblies of actin in cultured cells are stress fibres, lamellipodia and filopodia. Actin filaments are assembled into networks in lamellipodia and into radial bundles in filopodia. Lamellipodia are thin veil-like extensions of the cell periphery and comprise of a planar meshwork of unipolar actin filaments whose fast-growing plus ends are oriented outwards. Depending on cell type and condition, the lamellipodium can vary in breadth from ~1 μm to 5 μm and can exhibit highly variable numbers of radiating bundles 0.1–0.2 μm in diameter and many micrometers long.

The lamellipodium tip engages protein complexes to drive actin polymerisation (288). Fibroblastic cells attached to a substrate extend lamellipodia and filopodia in the direction of cell locomotion. Although much of what is known about the dynamics of the actin cytoskeleton in vertebrate cell shape development comes from studies of cells spreading, moving and associating in tissue culture — an essentially artificial environment, yet it is expected that the general principles in culture are also be applicable in vivo. Cell migration is modulated by a variety of factors such as regulatory peptides, metabolic substrates, cytokines, integrins, the extracellular matrix and polyamines. Cell culture is dependent on proper anchorage of cells to the growth surface. Poly-L-lysine is commonly used as adhesive molecule. Other biological molecules used to coat surfaces on which cells grow include fibronectin, laminin and other polyamines. Alternatives to these molecules such as

electropolymerization of different polymers (289) or growth on polyacrylamide films (290) has also being tested.

Spreading of fibroblastic cell types like Swiss 3T3 has been studied on PLL coated surfaces in order to elucidate the molecular mechanisms of how different patterns of actin cytoskeletons are determined (291). Migration and invasion assay platforms are well established and readily available through companies such as BD Biosciences and Chemicon. These assay platforms help study chemotaxis in cells and factors that affect cell motility.

In 2001, Nakagawa et al. showed that lamellipodia were induced in 3Y1 cells by culturing these rat fibroblasts on poly-L-lysine substrates (280). This led to the loss of the characteristic fibroblastic morphology and acquisition of a round phenotype of the 3Y1 cells on PLL treated coverslips. Similar results were obtained in studies done using other cell types such as transformed NIH3T3 cells grown on poly-D-lysine (292). Hence, this phenotype was chosen in order to test whether the surface of PP foils allowed cells to react to the chemicals coated on its surface.

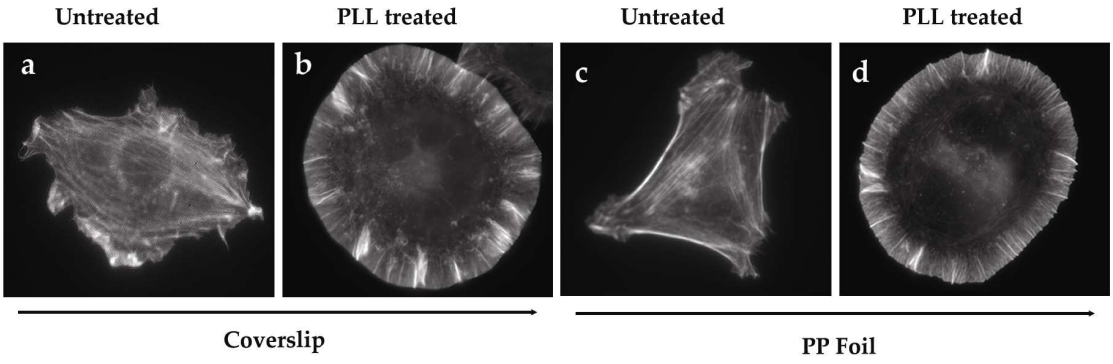

Figure 3-4. Repetition of cellular phenotypes on polypropylene surfaces
Morphology of 3Y1 cells grown on coverslips (b) as compared to those grown on patches (d) coated with poly-L-lysine (PLL). Panels (a) and(c) serve as the respective untreated controls. Single cells are photographed at a magnification of 630X with oil.

The 3Y1 cells seeded on to the patches treated with poly-L lysine depicted the characteristic round morphology as shown by Nakagawa et al. in 2001 (as seen in figure 3-4, see section 2.2.23.1 for detailed procedure). This required some optimisation with respect to the molecular weight of PLL, time for coating the patches and time point of observation of cells. According to Nakagawa et al., the coverslips were coated for 30 min and the lamellipodia formed were observed after plating the 3Y1 cells on the coverslips as early as after 1 h. For

the patches, it was observed that 3 h of coating with PLL and overnight growth of the cells on the patches gave the best results. Molecular weight of PLL used was 150,000 to 300,000.
As in the case of all surfaces, with a little optimisation the PP foils can be used quite well for cell based assays. Nevertheless, the reproducibility of this phenotype on PP surfaces proved that this surface allows the cells to show special phenotypes as seen on glass (coverslip) surface. And hence, these foils were further tested for the reproducibility of phenotypes in patches with fully synthesised peptides.

3.3 Inhibition of cytokinesis by the DT -2 peptide

Another fairly easy to observe cellular event that has been extensively studied is cytokinesis. Cytokinesis is the final event of the cell cycle and is the process that divides one cell into two daughter cells. Anti-mitotic compounds such as Disorazol A1 have the ability to inhibit tubulin polymerisation and thus have cytostatic activities (293). Anti-mitotic therapies that target tubulin are known to be effective and widely used to treat cancer. Anti-mitotic drugs such as Docetaxel and Paclitaxel act by stabilizing tubulin whereas others such as Vinblastulin act by de-stabilizing tubulin (294). In this study, peptides that inhibit cytokinesis were examined. These would be synthesised on the patches and presented to cells grown on the patches.
The sequence LRK5H, was identified by iterative deconvolution of every amino acid as a peptide sequence with the highest binding affinity for cGMP–dependent protein kinase Iα (cGPK). This sequence when fused to the membrane translocation signals from HIV-1 tat protein forms the DT -2 peptide (YGRKKRRQRRRPP–LRK5H) providing an efficient method for intracellular delivery (281; 295). The DT -2 peptide decreased NO-induced dilation in pressurised cerebral arteries. This peptide was also checked in proliferative cells (unpublished results Dostmann W. R. G., Sasse F. and Tegge W.) and was found to inhibit cytokinesis. A simple yet prominent morphological phenotype induced by peptides was required to test for cell growth on full length peptides. Hence, this peptide was used to test whether the compounds that were synthesised on the patches could induce the same morphological phenotypes they were expected to as when they were added externally to mammalian cells.

PtK2 cells (Rat kangaroo kidney epithelial cells) were tested on PP foils synthesised with the DT -2 peptide (synthesis done by U. Beutling). The DT -2 peptide is a highly charged peptide. Therefore, the Pep-1 peptide having the sequence Ac-KETWWETWWTEWSQPKKKRKVC(SH)- amide (also highly basic) was chosen as a negative control. The Pep -1 peptide mediates protein import of β –Gal (296). Both the D and L amino acid forms of the DT -2 peptide were externally added to the cells growing on non – synthesised patches as positive controls. The synthesised DT2 peptide was designed to release into cell culture media because of the ability of the carboxy-Frank-linker used here to be cleaved by solutions having neutral pH (see section 2.2.18). PP foils with amino functions and Jeffamine 130 were coupled with the linker preactivated with HOBt and DIC. After spotting of the linker solution on the foil, the DT-2 peptides were coupled and peptide synthesis was as done in section 2.2.17. The foils with the DT-2 peptide synthesised on them were used for cell based assays. These foils were first washed with ethanol and PBS pH 6.5. PtK2 cells were added to each patch and were allowed to grow on the patches for 2 days at 37°C. Thus the DT-2 peptide was available to the cells grown on the patches on the addition of cell culture media (DMEM). After this, the patches with cells were washed with PBS and fixed with 37% formaldehyde for 10 min. The cells on the patches were stained using anti-tubulin antibody and detected using a fluorescence microscope.

The cells were treated with the DT -2 peptide for 2 days and then observed. Treatment with the DT-2 peptide (both synthesised as well as externally added) resulted in cells where the inhibition of cytokinesis caused cells to have more than one nucleus (predominantly 2 nuclei but at times up to 4 nuclei in cells). This effect was also observed in both the externally added D- and L- DT -2 peptides. In each case, the cells were compared to cells growing on coverslips. The cells treated with Pep -1 peptide did not show any inhibition of cytokinesis (figure 3-5a). These cells were similar to untreated cells grown on coverslips and patches.

The foils were thus successfully used for the synthesis of small molecules; in this case peptides. In addition, the loading of the peptides on the patches was sufficient to see a phenotypic effect of these on cells. Hence, these conditions can be used as initial conditions for optimising any further assays performed with either random or directed screening of compound libraries synthesised on these patches.

Further optimisation of delivery of peptides in general presented to cells in this manner is possible by synthesising peptides that are capable of penetrating the cell membrane. Such

peptides are called protein transduction domains (PTD) or cell permeable peptides (CPP). They are derived from naturally occurring proteins such as anti-DNA mouse immunoglobulin (297; 298), DNA binding domain (homeodomain) of Drosophila transcription factor Antennapedia (299), the HSV-1 structural protein VP22 (300) and the Tat protein of human immunodeficiency virus type 1 (HIV-1) (301; 302) or artificially designed peptides (303). Such modifications can be incorporated into the synthesised peptides for a more efficient delivery into cell based assays.

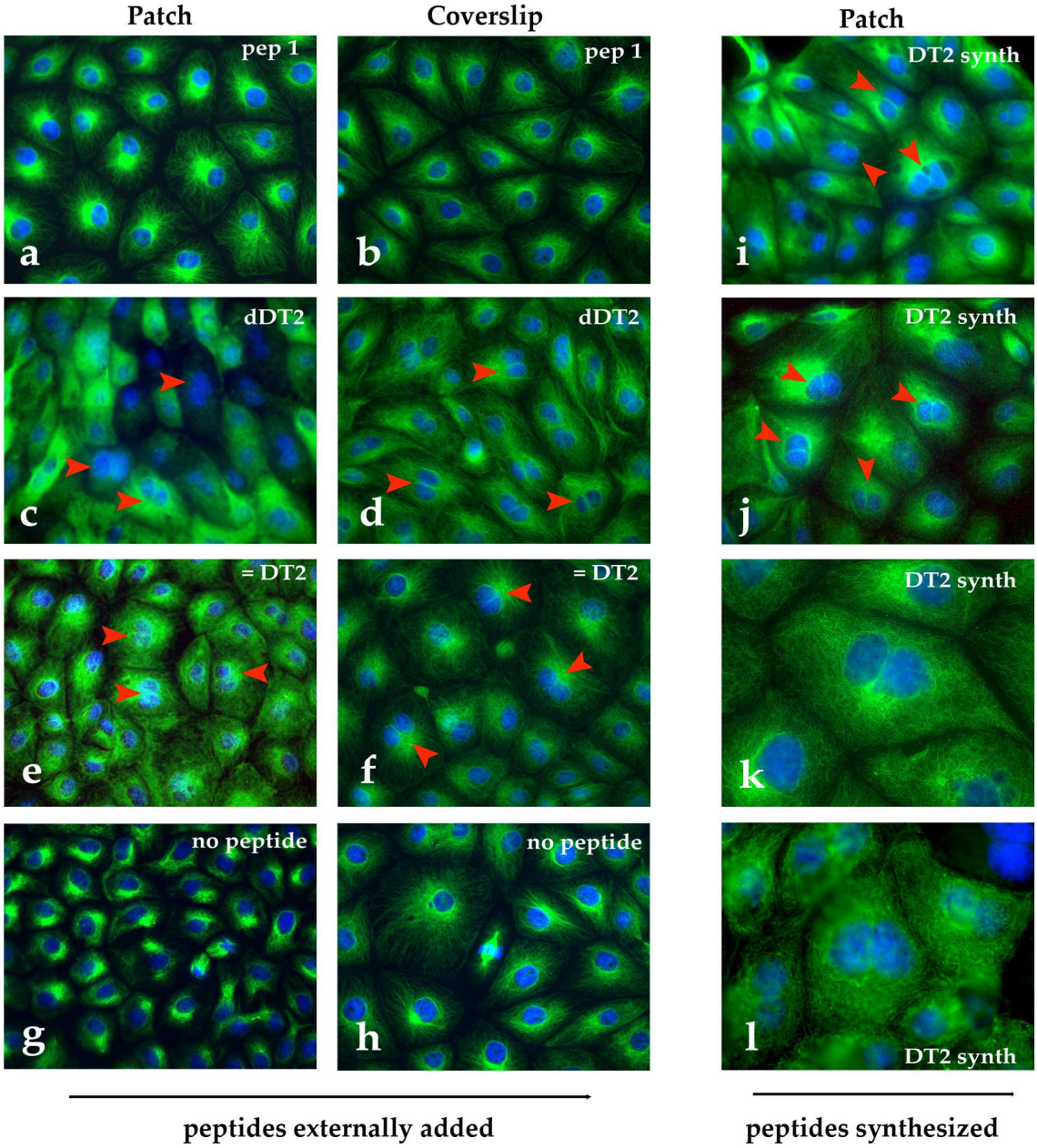

Figure 3-5. Effect of the DT -2 peptide on PtK2 cells.
The panel to the left shows PtK2 cells grown on the patches (a,c,e,g) and on the coverslip(b,d,f,h)s which were treated with externally added peptides. The panel to the right (i,j,k,l) shows patches with the DT2 peptide synthesised on which PtK2 cells have been grown. Cells grown with the Pep 1 control peptide externally added to patches and coverslips (a,b) showed no effect and were similar to

those that were untreated (g,h). On the patches synthesised the DT2 peptide is present as both D and L amino acids externally added. In the control (a,bg,h) all the cells have a single nucleus. In the cells grown on patches with the DT2 peptide or the patches or coverslips where the DT2 peptide is externally added, the presence of cells with two or more nuclei is observed, as pointed out with the red arrowheads. Magnification: a to j are 400X and k,l are 1000X (oil).

3.4 Fluorescence based readouts for cell based assays based on La

In order to test for the cleavage of the La protein inside a cell, a sensitive yet efficient method was required. The La protein was cloned into a cygnet vector based on fluorescence resonance energy transfer with the aim to exploit changes in FRET signal by the intended induction of La cleavage. This would enable the rapid screening of compounds using changes in emission spectra of cells transfected with the cygnet La protein.

Fluorescence resonance energy transfer (FRET) is a non-radiative process where energy from a donor is transferred to an acceptor when they have overlapping emission/ absorption spectra with a suitable orientation and distance (in the range of 10–100 A°). FRET was described about 60 years ago (304) and enables the study of molecular interactions with an optical microscope well beyond the resolution limit of light microscopy. This phenomenon occurs when the proximity of a suitable pair of spectrally distinct fluorescent molecules is sufficiently close (between 1 and 10 nm) for a radiationless transfer of energy via a dipole-dipole interaction from the excited fluorophore (donor) to the second fluorophore (acceptor). Since transfer efficiency is dependent upon distance between the acceptor and donor, this technique is inherently sensitive to small physical changes in proximity. Förster distance, defined as the distance between the donor and acceptor where the average efficiency of energy transfer is 50%, is used when estimates of intramolecular distances between molecules are desired. The principles of FRET have been reviewed in detail by Pollok and Heim (305).

Green fluorescent protein (GFP) is a spontaneously fluorescent protein which has been commonly adopted as an excellent reporter module of the fusion proteins (306). An important feature of GFP is that variants of GFP have showed distinguished spectral properties which can be used as donors and acceptors of FRET (307; 308). The original pair of fluorescent proteins was a blue fluorescent protein (BFP) donor and a GFP acceptor with

relatively low quantum yield, easy bleaching, and high autofluorescence background (308). To overcome these drawbacks, a pair of GFP mutants with longer wavelengths, namely cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), has been reported with improved FRET efficiency (307; 309-311).

It is possible to study both intramolecular FRET or intermolecular FRET by fusing two spectrally overlapping GFP variants to two different regions of the same protein or to two different proteins facillitating the study of protein-protein interactions or protein conformation changes (312). Commonly, FRET is employed to visualise protein-protein interactions by targeting the desired cellular constituents with fluorescent dyes, fluorescent antibodies and/or fluorescent proteins. A cellular target is labeled with a donor fluorophore while another is labeled with an acceptor fluorophore. If the fluorophore labeled proteins are intimately associated (Fig. 3-6), excitation of the donor molecule will produce an emission at the acceptor wavelength. (Periasamy and Day, 1999; Sekar and Periasamy, 2003).

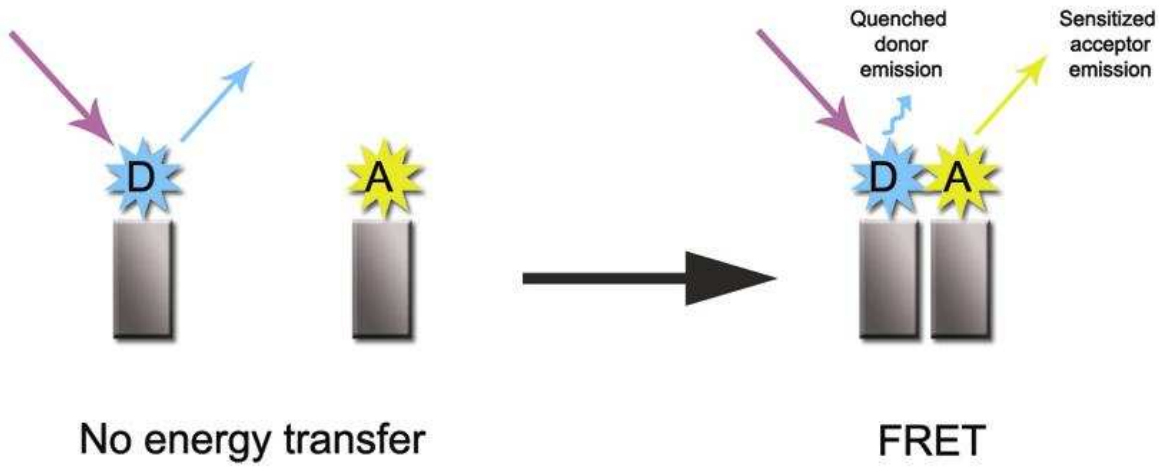

Figure 3-6. FRET schematic using CFP and YFP fusion proteins.
No energy transfer: Excitation of a donor fluorophore (D) sufficiently separated from its acceptor fluorophore (A) will result in only donor emission.
FRET energy transfer: Excitation of a donor fluorophore (D) within approximately 10nm of its acceptor fluorophore (A) will result in acceptor emission and quenched donor emission.

The development and use of sensors based on the genetically encoded variants of green-fluorescent proteins has facilitated the observation of 'live' biochemistry on a microscopic level, with the advantage of preserving the cellular context of biochemical connectivity, compartmentalization and spatial organization. Protein activities and interactions can be

imaged and localised within a single cell, allowing correlation with phenomena such as the cell cycle, migration and morphogenesis (313; 314). Although new GFP variants are being created to act as better FRET pairs (170; 315; 316), CFP and YFP have been used commonly as FRET pairs in a variety of studies as can be seen in the table below.

	Application	**FRET pair used**	**Reference**
Kinases	cAMP indicator in cardiac myocytes and transgenic fruit flies	CFP- YFP	(317; 318)
	Calmodulin based Ca2+ binding	ECFP -EYFP	(319; 320)
	GMP related neural studies	BFP- GFP	(321)
	Protein phosphorylation	CFP- YFP	(322)
	Signal transduction pathways	CFP - YFP	(323)
Cell based	Cell motility	CFP- YFP	(324)
	Cell membrane lipid rafts	CFP- Fast DiI	(325)
	Nuclear transport Vesicle transport	ECFP- EYFP CFP- YFP	(326) (327)
	Hormonal studies Growth factors	UCP-oyester 556 fluorescein/rhodamine and Cy3/Cy5	(328) (329)
Immunological responses	Study structures of biomolecules involved in malignancy of tumours	CFP-YFP Alexa Fluor 594 hydrazide & Alexa Fluor 488-SE	(330) (331)
	Viral/pathological studies	EBFP- GFP ECFP-EYFP	(332) (333)
Cancer	Tumour diagnosis	7-aminoactinomycin D Hoechst 33258	(334)
	Apoptotic signalling	CFP- YFP CFP-YFP-mRFP	(335) (336)
Diseases	Alzheimers	CFP- YFP	(337)

Table 3-1. Examples of biological studies based on FRET

3.4.1 FRET spectra *in vitro*

3.4.1.1 Sorting of cygnet La transfected HeLa cells

The cloning of the wildtype La in to the cygnet vector was as described in section 2.2.27. This vector was kindly provided by Dr. W. Dostmann from the University of Burlington, Vermont, USA (275). Cloning was done in the lab of Dr. Tilman Heise at the HPI in Hamburg.

HeLa cells transfected with the cygnet La plasmid were sorted in order to separate the transfected cells from the non-transfected cells. This was done based on the parameters for the YFP fluorophore. The excitation laser used was 488 nm. The cells were sorted twice for enrichment and purification respectively. The transfection efficiency attained was between 8% to 18% and the samples contained about 80% transfected cells after sorting. In the figure 3-7 below, region R3 represented the transfection efficiency. If needed, depending on the transfection efficiency, the cells were also subjected to an extra step of enrichment prior to the purification step.

These sorted cells could not be maintained for more than three days in culture as they gave up the Cygnet La plasmid and became non-fluorescent cells in culture. On sorting, they were lysed and used for *in vitro* FRET studies in order to confirm that the Cygnet La protein on protease treatment displayed no FRET.

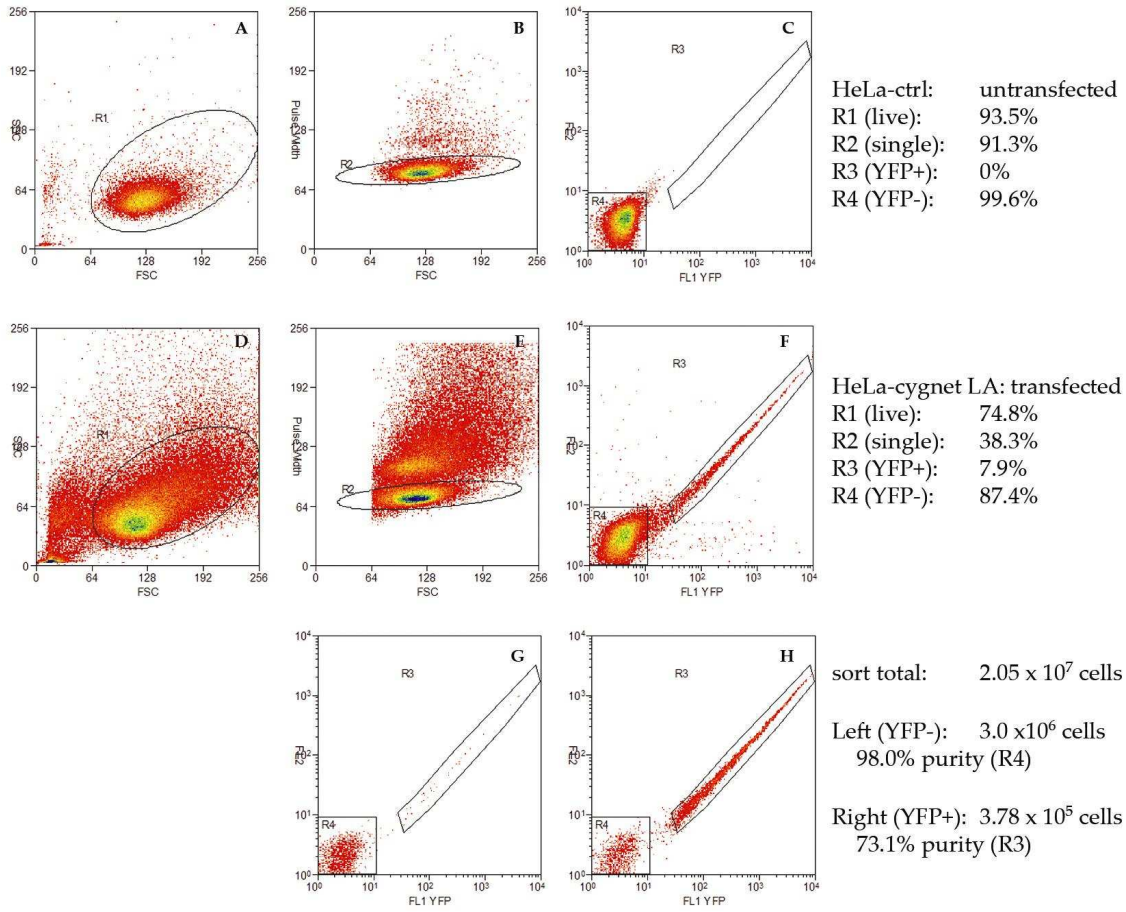

Figure 3-7. Sorting of Cygnet La transfected HeLa cells
(A-C) FACS sorted untransfected control Hela cells, (D-H) FACS sorted Hela cells transfected with the Cygnet La plasmid. Region R1 (in A and D) represent the number of live cells. Region R2 (in B and E) represents the number of single cells. R3 and R4 represent the YFP positive and YFP negative cells respectively. G and H represent cells from the second sort. The transfection efficiency in this experiment is 7.9% (region R3 in F). A total of 3.78 x 10^5 cells are obtained for further in vitro FRET experiments after this FACS sorting.

3.4.1.2 Protease mediated cleavage of the Cygnet La protein

La is predominantly a nuclear protein. On transfection into mammalian cells, cygnet La is retained in the cytoplasm. This is most likely due to the cloning of the GFP molecule at the C terminus of the La protein. Cygnet La is probably not localised into the nucleus because the GFP masks the nuclear localisation signal (NLS). Despite this, the La protein obtained after transfection was fully functional and gave FRET readings *in vitro*. FACS sorting aided in further concentrating the cell lysate, since owing to the absence of a purification tag in this vector it was not possible to purify the La protein using affinity binding.

Also, it was critical to mimic the conditions inside the cell where the La protein may be acted upon by proteases to trigger cleavage of the protein. One way to initiate this kind of

cleavage is through the induction of apoptosis, known to trigger the intracellular translocation of the La protein.

In this study, this was reproduced *in vitro* using proteases to cleave the La protein (284). Treatment with Chymotrypsin (CT) yielded 3 fragments that were visible on the gel after silver staining but were no longer recognised by the anti–La antibody 4B6. Elastase and Proteinase K (PK) cleaved the WT La into smaller fragments with at least one fragment still recognised by the primary antibody 4B6 (figure 3-8).

This was then repeated in Cygnet La using Elastase at 5 μg/ml and 90 μg/ml. These reactions were then stopped at 10 min and 30 min respectively in order to find the right conditions for Cygnet La cleavage (figure 3-9). Digestion with Elastase at 10 min did not show significant digestion at 5 μg/ml and 90 μg/ml, however cleavage was observed at 30 min.

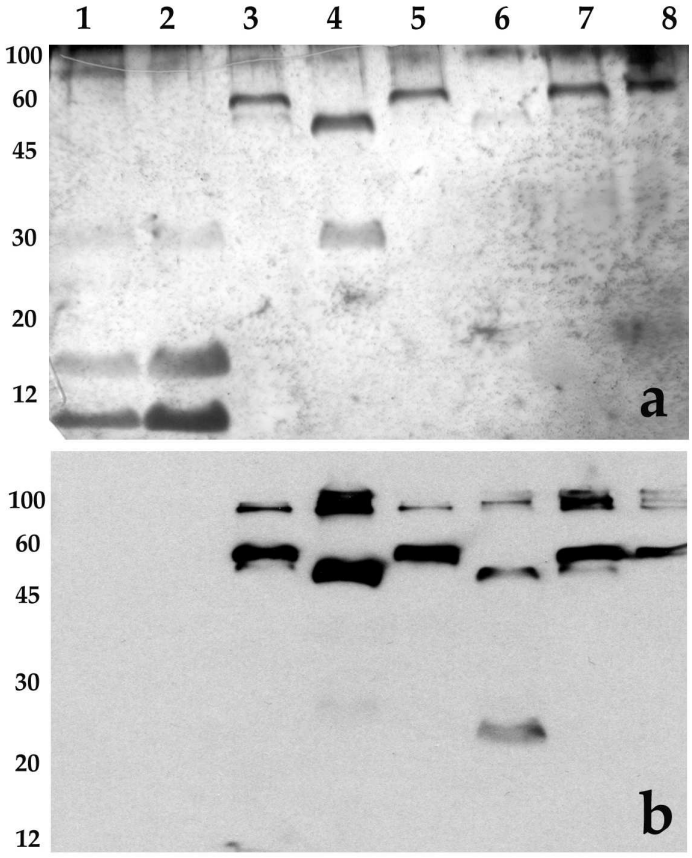

Figure 3-8. Controlled protease digestion of wildtype La protein to check for the cleavage pattern
Lane 1: Chymotrypsin 25μg/ml, lane 2: Chymotrypsin 50 μg/ml, lane 3: Elastase 5 μg/ml, lane 4: Elastase 90 μg/ml, lane 5: Proteinase K 0.6 μg/ml, lane 6: Proteinase K 5.4 μg/ml, lane 7: Proteinase K 1.8 μg/ml, lane 8: wildtype La 1.5 μg. Digestion with proteases was done for 30 min. a) Silver staining of 10% SDS PAGE Gel. b) ECL after binding the blotted membrane with primary antibody 4B6 and secondary antibody G anti-mouse conjugates with HRP.

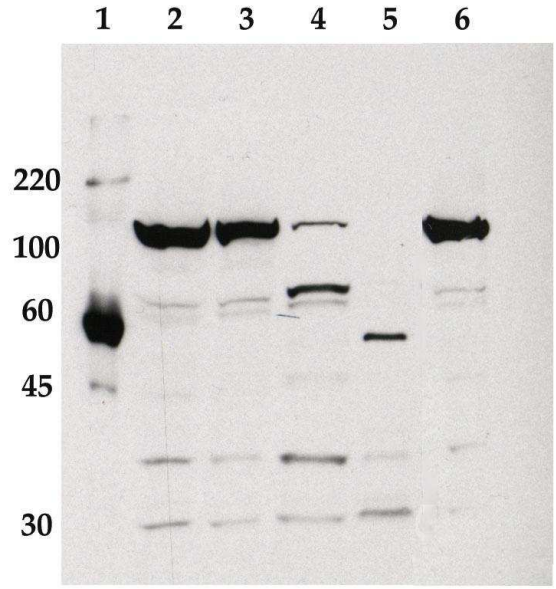

Figure 3-9. Elastase digestion of the Cygnet La protein
Elastase digestion of the Cygnet La protein to check for the cleavage pattern in 10 min and 30 min lane 1: Ladder, lane 2: Elastase digestion done at 5µg/ml at 10 min, lane 3: Elastase digestion done at 90µg/ml at 10 min, lane 4: Elastase digestion done at 5µg/ml at 30 min, lane 5: Elastase digestion done at 90µg/ml at 30 min, Lane 6: Cygnet La protein undigested.

Further, to check for possible cleavage sites in the individual fluorophores for Elastase, a time dependent cleavage was carried out and the blots after Western blotting were bound with anti-GFP antibody and detected by ECL. In order to check that the protease did not cleave the fluorophores, a time dependent protease cleavage of the cell lysate obtained from EYFP and ECFP vectors was done (from clonetech). Lysate from Hela cells transfected with either of these plasmids was subjected to Elastase digestion without cell sorting. Further experiments were done as in the case of the Cygnet La transfected HeLa cell lysates. It was observed that the fluorophores did not get cleaved over time with Elastase while the Cygnet La protein did. This can be seen in the following figures. The primary antibody used in this case was the anti-GFP antibody to detect for cleavage of the fluorophores.

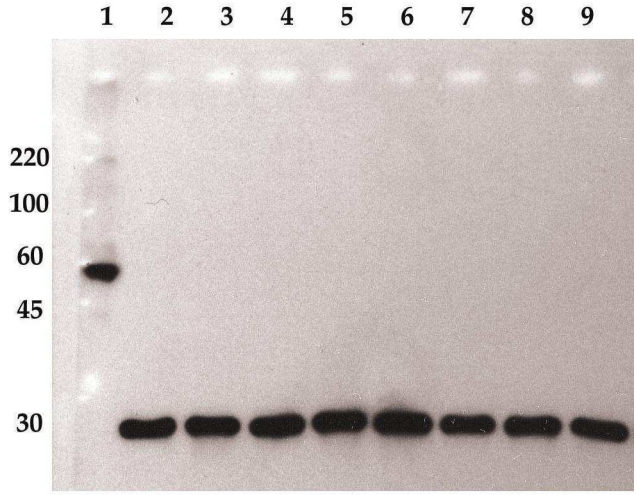

Figure 3-10a. Elastase digestion of the ECFP protein
ECL on binding with anti-GFP antibody after ECFP Elastase (ELS) digestion
Lane 1: Ladder, ECFP ELS digestion at time points lane 2: 0 min, lane 3: 1 min, lane 4: 2 min, lane 5: 5 min, lane 6: 10 min, lane 7: 15 min, lane 8: 30 min, lane 9: 60 min.
The band in lane 1 is the 50 KDa band from the chemichrome western control ladder from Sigma.

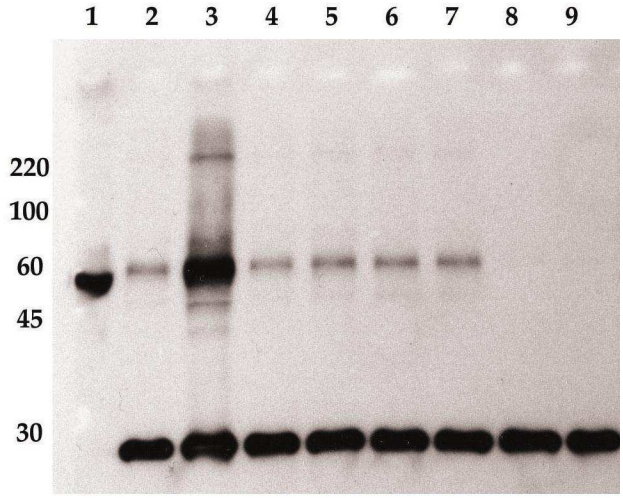

Figure 3-10b. Elastase digestion of the EYFP protein
ECL on binding with anti-GFP antibody after EYFP ELS digestion
Lane 1: Ladder, EYFP ELS digestion at time points lane 2: 0 min, lane 3: 1 min, lane 4: 2 min, lane 5: 5 min, lane 6: 10 min, lane 7: 15 min, lane 8: 30 min, lane 9: 60 min.
The band in lane 1 is the 50 KDa band from the chemichrome western control ladder from Sigma.

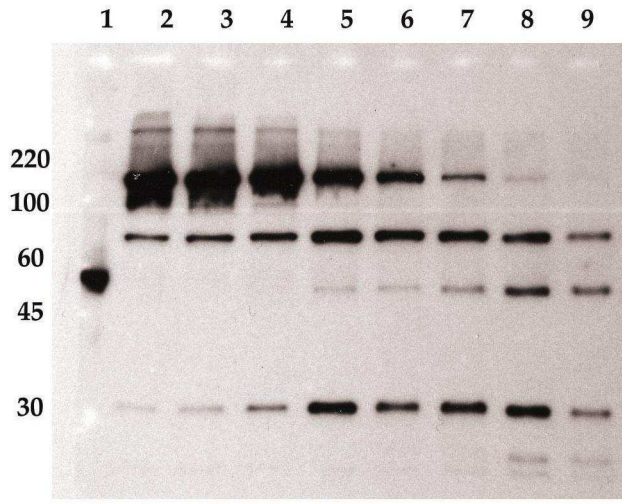

Figure 3-10c. Elastase digestion of the Cygnet La protein
ECL reaction after Cygnet La digestion with ELS
Lane 1: Ladder, Cygnet La ELS digestion at time points lane 2: 0 min, lane 3: 1 min, lane 4: 2 min, lane 5: 5 min, lane 6: 10 min, lane 7: 15 min, lane 8: 30 min, lane 9: 60 min.
The band in lane 1 is the 50 KDa band from the chemichrome western control ladder from Sigma.

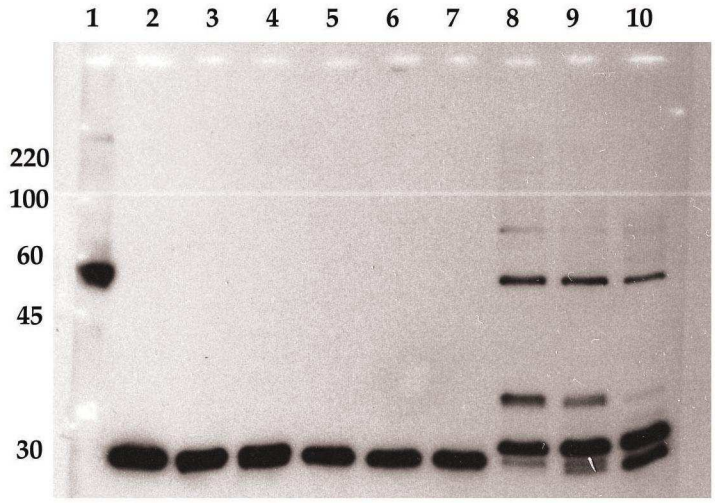

Figure 3-10d. Later time points of Elastase digestion of ECFP, EYFP and the Cygnet La protein
ECL on binding with anti-GFP antibody after ECFP ELS digestion (lanes 2 to 4)
EYFP ELS digestion (lanes 5 to 7) and Cygnet La digestion (lanes 8-10)
Time points 120 min, 240 min, overnight in each case
The band in lane 1 is the 50 KDa band from the chemichrome western control ladder from Sigma.

These studies thus indicate that the Cygnet La protein is digested using the protease Elastase which does not digest the ECFP or the EYFP fluorophores themselves. Thus we were able to mimic the in vivo cleavage of the La protein by proteases.

3.4.1.3 Spectra from the digested Cygnet La lysates

The lysate from the sorted HeLa cells (transfected with the cygnet La plasmid) was read in a spectrofluorimeter and the sample was excited with 430 nm for ECFP. Emission spectra were recorded in quartz suprasil cuvettes for 400 µl of lysed cell samples at excitation 430 nm for ECFP in the Fluoromax 3 fluorometer. The emission spectra were read from 460 nm to 560 nm, which would cover the range for ECFP and EYFP emission. The first peak obtained at around 470 nm is the emission from the ECFP fluorophore and the peak at around 530 nm is from the energy transfer mediated excitation of the EYFP fluorophore. The spectra were exported from the Instrument Control Center software and plotted in EXCEL for further comparative analysis.

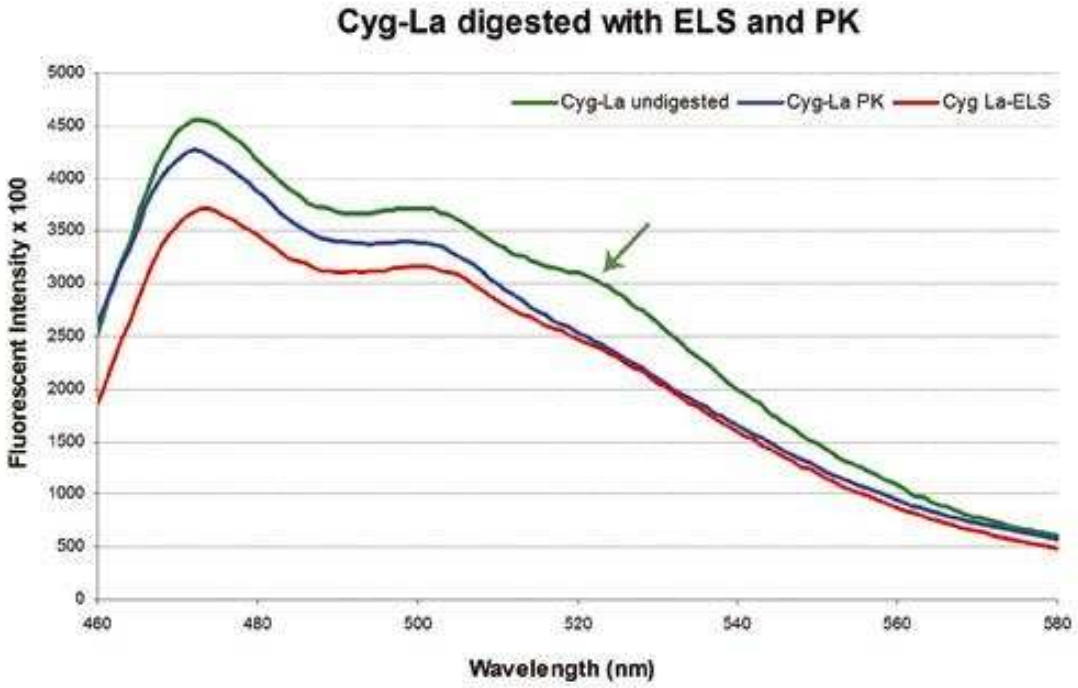

Figure 3-11. Spectra obtained before and after treatment of Cygnet La in HeLa cell lysates with proteases
Spectra obtained on excitation of the Cygnet La at 430 nm. The first peak obtained at around 470 nm is the emission from the ECFP fluorophore and the peak at around 530 nm is from the energy transfer mediated excitation of the EYFP fluorophore. The spectra in green are from the undigested Cygnet La from lysed transfected Hela cells. The spectra obtained after both Proteinase K digestion (in blue) and Elastase digestion (in red)

The cygnet La lysate was also digested with Elastase and Proteinase K and the spectra were recorded again. The peak at 530 nm that could be observed in the undigested sample

disappeared after controlled protease digestion. These results are in accordance with the expected results on the cleavage of cygnet La.

3.4.2 FRET spectra *in vivo*

One of the techniques for measuring FRET is acceptor photobleaching: the increase in donor fluorescence after complete acceptor photobleaching is a measure of the FRET efficiency. It is often used as a relatively straightforward approach for determining FRET with conventional and confocal microscopy (338; 339). When FRET pairs are in close proximity, the donor energy is transferred to the acceptor molecule. Bleaching of the acceptor fluorophore will dequench the donor and result in increased donor emission (as depicted in figure 3-12).

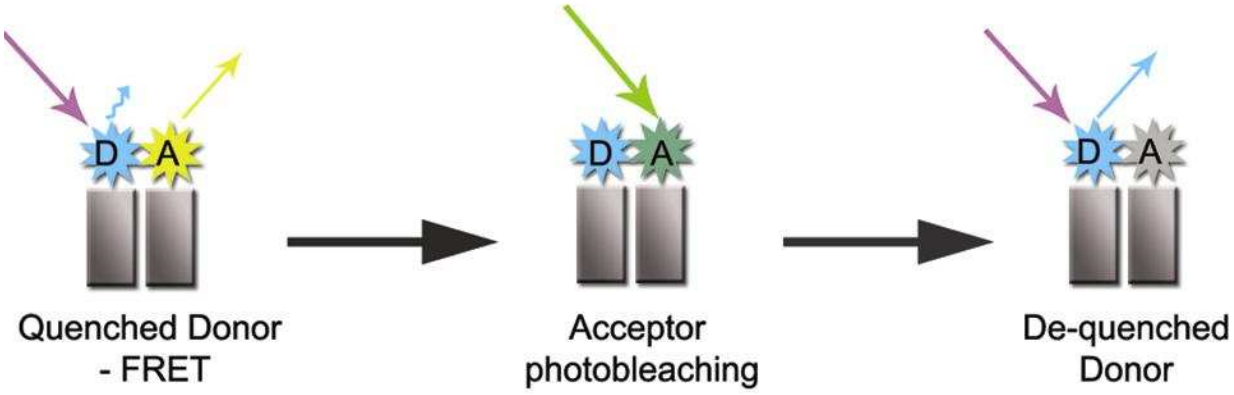

Figure 3-12. Simplified schematic of how acceptor photobleaching works.
Quenched Donor (FRET): Excitation of a donor fluorophore that is (D) within 10 nm of its acceptor fluorophore (A) will result in acceptor emission and quenched donor emission.
Acceptor Photobleach: Sufficient excitation to irreversibly photobleach the acceptor fluorophore (A) eliminates the potential for the acceptor to participate in energy transfer.
Dequenched Donor: Excitation of the donor fluorophore (D) following acceptor photobleaching (A) will result in dequenched donor emission.

When energy transfer occurs, the donor emission will be quenched because of the direct transfer of excitation energy to the acceptor. If the acceptor fluorophore is destroyed, FRET will be eliminated and the donor signal will be de-quenched. The increase in the donor signal resulting from de-quenching is a direct measure of the efficiency of FRET. The technique of acceptor photobleaching FRET (apFRET) exploits this characteristic, and measures the de-quenching of the donor signal in the regions of the cell where FRET had occurred. The apFRET method requires the selective bleaching of the acceptor, because any

bleaching of the donor fluorophore will lead to an underestimation of the de-quenching. Further, bleaching of the acceptor should be as close to completion as possible, since any remaining acceptor will still be available for FRET, again resulting in an underestimation of the donor de-quenching. One of the many advantages of FRET is that it is robust yet sensitive enough and can be miniaturised to be applied for high through-put based drug screening.

Cygnet La transfected HeLa cells were excited with light of wavelength 458 nm and emission spectra were recorded from 467 to 628 nm in order to look for FRET. Acceptor photobleaching with the 514 nm laser resulted in bleaching of the EYFP molecules. This can be clearly seen by comparing figure 3-13.

In figure 3-13b, the second peak at 530 nm is reduced due to photobleaching of the fluorophore. However, the control cell, where there is no photobleaching shows the same amount of EYFP intensity both before and after photobleaching, thereby serving as an internal control also against possible leakage of photobleaching or carrying over to the next cell. Emission spectra were again recorded for the defined region as well as for the whole cell (R3 and R2 respectively). At the same time, there is a visible increase in intensity of the ECFP after photobleaching depicting dequenching of the donor. The regions R2 and R3 both show an increase in the ECFP signal after photobleaching in figure 3-11. However the increase in the donor emission after photobleaching is still weak. This is proof of a weak FRET signal.

Hence, FRET can be used *in vivo* as a method to screen for compounds that cleave the La protein. However, the cygnet La construct should be optimised further for a stronger FRET readout. This would be essential for high throughput screening assays where the intensity of the FRET signal could be a deciding factor for accuracy of the various automation steps. The length of the adjoining fragments between the La protein and the fluorophores should be modified to start with. Further quantification based on acceptor photobleaching can be done by calculating the FRET efficiency (339). Though this assay needs further optimisation on the patches for random library screening, these initial steps confirm that the cygnet La vector is functional and can be adapted for high through put screening.

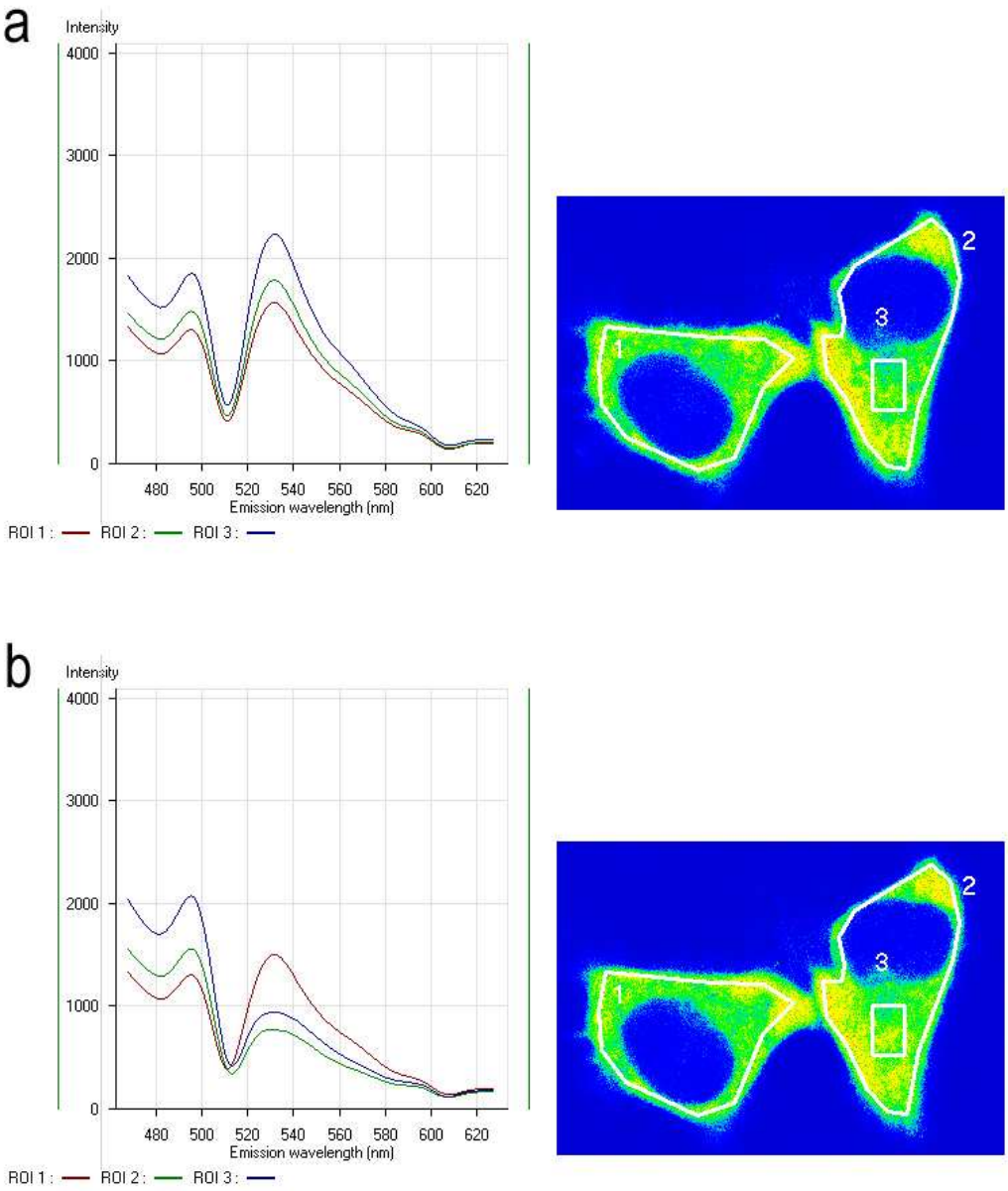

Figure 3-13. ***In vivo*** **FRET changes observed in HeLa cells on acceptor photobleaching**
Two Hela cells transfected with the Cgynet La plasmid are depicted in right panels. The left panels depict the spectra obtained from regions of interest within these cells.
(a) Region ROI 1 depicts the entire cell 1 used as a control cell in this experiment. It is not subjected to acceptor photobleaching. Spectra from this region are in red. They remain unchanged both in panels a and b.
(b) Region ROI 2 is drawn to include the entire experimental cell 2 (as shown in panel b). Acceptor photobleaching is done in ROI 3 in this cell. Spectra from ROI 2 (entire cell) are non green and spectra from the photobleached region ROI 3 are in blue. Both these spectra are increased in the emission range of the donor and reduced in panel after acceptor photobleaching in the emission range of the acceptor.

3.5 Alternative GFP La strategy

3.5.1 Cloning of an NES into GFP-La vector

The cygnet La FRET strategy for cell based assays for the screening and identification of compounds capable of triggering protease activities within a cell to mediate La protein cleavage as described in section 3.4 worked well both *in vivo* as well as *in vitro*. However, for reasons unknown, the cygnet La protein remained localised in the cytoplasm. It has been reported that upon induction of apoptosis, the La protein was cleaved by proteases (340). An assay that would allow the translocation of the La protein from the nucleus to the cytoplasm upon cleavage would enable the simple cellular event of translocation to be used as readout for the screening of compound libraries.

WT La protein had been cloned in the pEGFP-N1 vector in Hamburg. Apoptosis induction experiments performed by Dr. Tilman Heise at the HPI in Hamburg didn't yield the expected change in intracellular translocation with this plasmid and so for this project, it was essential to clone a nuclear export signal (NES) into this plasmid. The pEGFP -N1 vector from Clonetech has a size of 4.7 kb and a Kanamycin resistance marker. The GFP WT La plasmid was linearised with Sac I restriction enzyme and the insert was cut using Sac I/Hind III double digest. The cloning strategy for the NES WT GFP La has been described in section 2.2.25. Cloning was attempted for all three nuclear export signals at the Xho I restriction enzyme site which lay between the GFP and the La sequences.

From the 3 NES clonings that were attempted (based on the speed at which they traffic their cargo), the REV NES sequence was successfully cloned into the Xho I site between the GFP molecule and the La protein DNA and this plasmid was used for the apoptosis induction experiments below. The E1 B cloning was not successful and the PKI cloning was less effective as compared to the REV NES cloning (data from Dr. Tilman Heise, HPI Hamburg).

3.5.2 Effect on compartmentalisation of the La protein in transfected A549 cells

Epothilones represent a novel class of anticancer drugs which inhibit the cell cycle and strongly influence cell division. The biological activity of epothilones is associated with their capacity to bind to the protein tubulin of microtubules and to disturb the dynamic

equilibrium between microtubule assembling and disassembling. Consequently, in dividing cells, it leads to mitotic arrest and to apoptotic cell death.

In taxane resistant tumours it is known to induce G2M arrest in many cell types. Exposure of human cancer cell lines to epothilone causes aberrant spindle formation during mitosis, which results in mitotic arrest at the metaphase/anaphase boundary and eventually apoptotic cell death (341). Studies with water soluble epothilone analogues such as BMS 310705 show that this class of compounds can induce significant apoptosis, decrease survival and utilise the mitochochondrial–mediated pathway for apoptosis (283).

The GFP NES La plasmid was transfected into A549 cells. Along side, the wildtype La plasmid was also transfected into A549 cells. The effect of treatment of these cells with epothilone on the cellular localisation of the La protein was studied. Epothilone is a known apoptosis inducer in mammalian cells (342) and has an IC-50 value of 0.23 ±0.05 nmole for net growth inhibition of A549 cells (342). La protein localisation was observed in the cytoplasm with cells having non-fluorescent nuclei in the case of treated NES WT La transfected cells. It has been shown that the La protein translocates into the cytoplasm on apoptosis induction (343). The same effect could be observed through this experiment in A549 cells. The GFP NES WT La and the GFP WT La untreated controls did not show this effect.

Ayukawa et al. have reported that the La protein was truncated on apoptosis induction and cleaved by caspase-3 or closely related proteases at Asp-374 in the COOH terminus to form a 43 KDa protein in HL-60 cells (340). In the EpoB induced apoptosis experiments, a complete translocation of the La protein from the nucleus to the cytoplasm was observed. Hence, the GFP NES WT La has an advantage over the WT La in the development of further screening assays using small molecules. With this assay now established, it will be possible to screen chemical libraries for compounds that can induce the cleavage of the La protein.

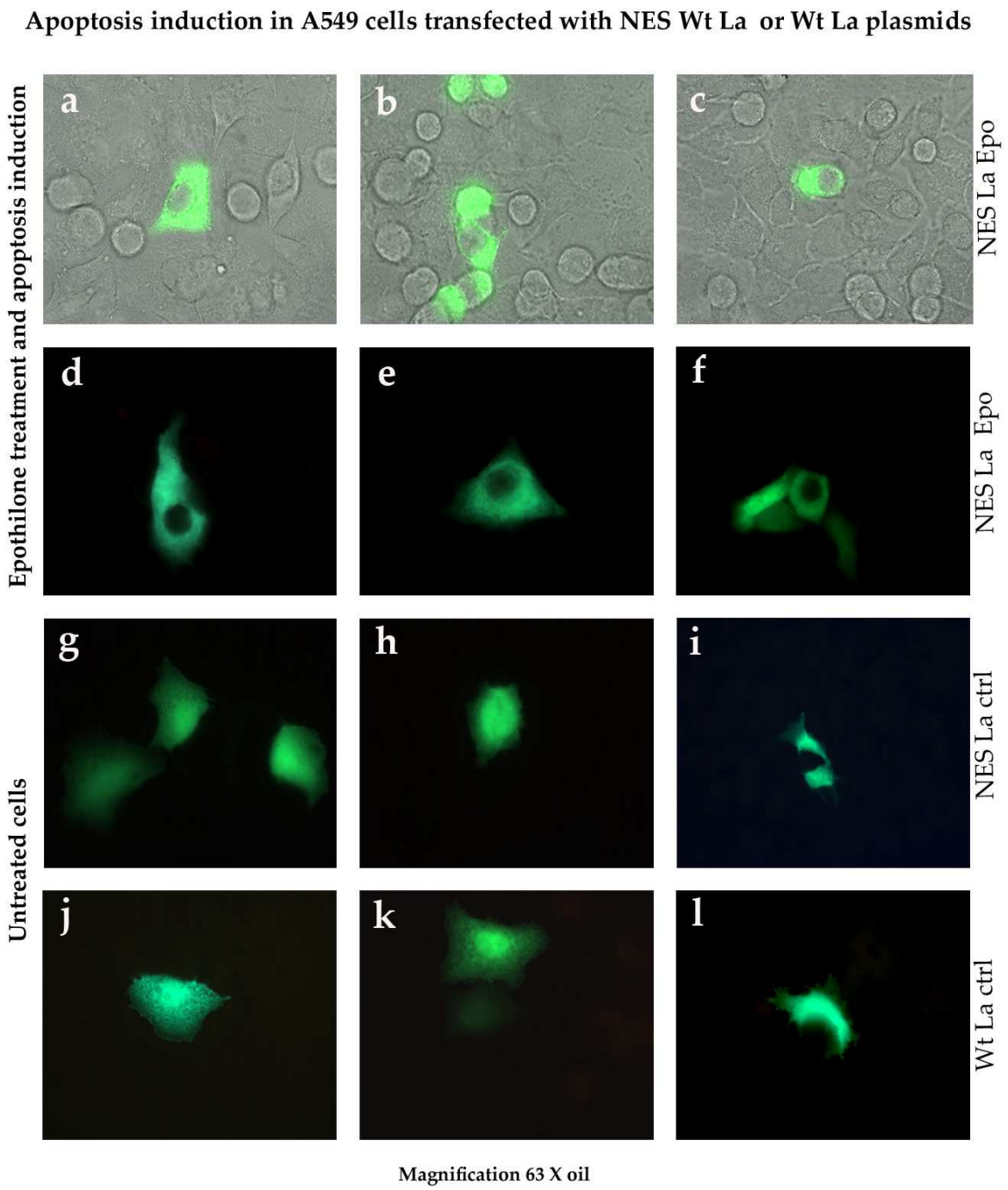

Figure 3-14. Effect of the induction of apoptosis on the intracellular localization of the La protein GFP NES La transfected A549 cells **show translocation of the GFP La protein in the cytoplasm** after treatment with epothilone (a-f). The GFP NES La control cells (g-i) as well as the GFP La (j-l) transfected A549 cells both show the distribution of the La protein predominantly in the nuclei.

3.6 Search for small molecule binders of the La protein

3.6.1 Binding assays with radioactively labelled proteins

Published work suggest that the La protein stabilizes the HBV mRNA by binding to the viral RNA. Horke et al. demonstrated that the RRM2 of La is required for binding of La to HBV RNA *in vitro* (272). Hence we postulate that a compound which binds specifically to the RRM2 might be able to block the interaction between La and HBV RNA. HBV RNA would be less protected and degraded. The aim of the following experiments was to establish a screening assays to detect peptides able to bind the La protein via the RRM2. The goal was to be able to detect sequences of small molecules that would not bind to the mutant La protein (Mut Δ2) but would bind to the wildtype La protein, thereby mimicking the binding of the La protein to the HBV RNA. The respective La proteins used for binding assays with libraries of small molecules needed to be easily detectable for comparison. One way to ensure this was to perform the assays with *in vitro* synthesised radioactively labelled proteins which would allow the quantification of the amount of bound protein.

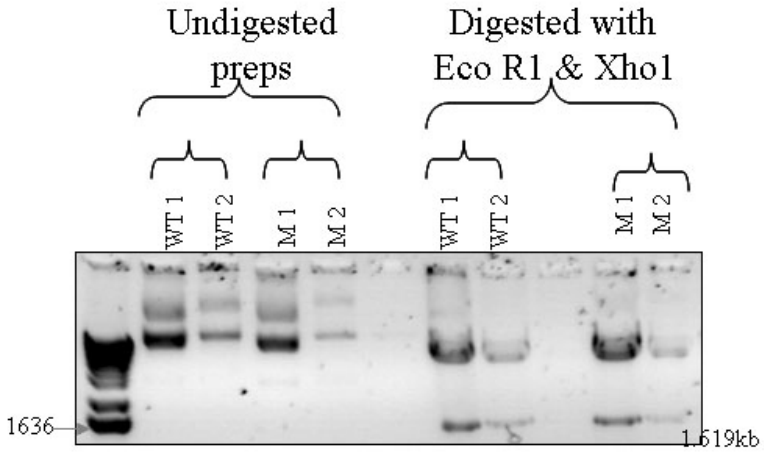

Figure 3-15. Wildtype (WT) La and Mutant (Mut) La plasmids after purification from E. coli DH5α
Lane 1: Ladder, Lanes 2 and 3: Wildtype La plasmid purified from two different transformed colonies (hence labeled WT1, WT2), Lanes 4 and 5: Mutant La plasmid purified from two different transformed colonies (hence labeled M1, M2), Lanes 7 and 8: Eco RI digested wildtype La, Lanes 10 and 11: Eco RI digested mutant La. The wildtype and mutant La plasmids have a size of 6.97 Kb and after Ecor RI/XhoI digestion the inserts have a size of 1.6 kb.

The wildtype and mutant La plasmids were cloned in the pET-28 vector having a Kanamycin resistant marker for growth in *E. coli*, a size of 5.37 kb and a T7 promoter. The

size of the plasmids which were purified from *E. coli* DH 5α was confirmed on 1% agarose gels. Also these plasmids were digested O/N with EcoR I to linearise the plasmid and with EcoR I/Xho I double digest to remove the insert. The inserts had a size of 1.6 kb. The undigested plasmids had a size of 7 kb on the gel. The plasmids were purified using the Qiagen spin columns according to the manufacturer's instructions and were further used for *in vitro* transcription & translation kit to produce radioactive proteins.

3.6.1.1 Probe preparation

The radioactive protein that resulted from the *in vitro* transcription & translation reactions was investigated on the gel. 2.0 µl of the reaction mixture was loaded on the gel. The gel was blotted and ECLs were performed for the identification of the La protein bands. Also afterwards the gels were exposed to X ray films and the radioactive signals emanating from the synthesised protein were investigated (figure 3-16). Thus although the quantities of recombinant La proteins produced were not sufficient for detection by ECL, autoradiography could detect these effectively.

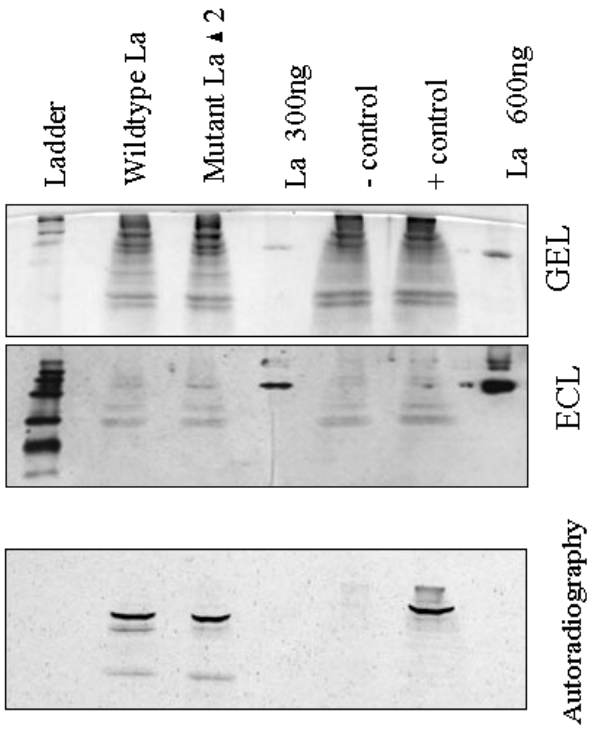

Figure 3-16. Radioactive probe for binding assays using SPOT membranes
Lane 1: Ladder, Lanes 2 and 3: Radioactive Wt La protein and Mut La Δ2 protein obtained from the *in vitro* transcription & translation kit, Lanes 4 and 7: purified recombinant control La proteins (300 ng

and 600 ng in Lanes 4 and 7), Lanes 5 and 6: negative and positive controls from the Promega *in vitro* transcription & translation kit. All reactions were performed according to the manufacturer's instructions. It is possible to detect the La proteins produced using the *in vitro* transcription & translation kit (in the autoradiography panel) and compare its size in the gel to the purified recombinant control La proteins (300 ng and 600 ng in Lanes 4 and 7 in the ECL panel).

3.6.1.2 Binding assay with RR membranes

SPOT peptide libraries of both L and D amino acids of hexamer and octamer peptides were screened with the La proteins to find possible binding partners. The aim was to find peptidic binding partners that could bind to the wildtype La and not with the mutant La. The binding assays were thus first done with the radioactive mutant La protein and after stripping of the membranes, were repeated with the wildtype La protein. After each stripping, the membranes were exposed to the Phosphorimager screens to check for effective stripping. The different libraries tried out are as listed below:

Starting Libraries screened	Sequence	aa residues in spots
Lib 17 LL	XXX12XXX	spot signals with Arginine and Lysine residues
Mixed 65 LL	XX12XX	spot signals with Arginine and Lysine residues
Lib 18 DL	XX34XX	No signals
Lib 18 DL	XXX45XXX	No signals
Lib 19 DC	XX34XX	No signals
Lib 19 DC	XXX45XXX	No signals
Lib 22 LC	different mers	Mutant spots similar to Wildtype spots in intensity

Table 3-2. Libraries screened using binding assays with the Mutant and Wildtype La proteins

The starting libraries screened and their sequences are listed. Also the defined amino acids of the spots obtained on binding are listed.
(**DL**:D aa linear, **LL**:L aa linear, **DC**:D aa cyclic, **LC**: L aa cyclic)

LL hexamer and octamer SPOT peptide libraries gave signals that had arginine or/and lysine in the known positions. However, binding assays with the LL octamer library resulted in spots that showed ''RR'' spots lighting up with more intensity as compared to the Mutant La binding assays when compared with AIDA and Corel Chart softwares (figure 3-17).

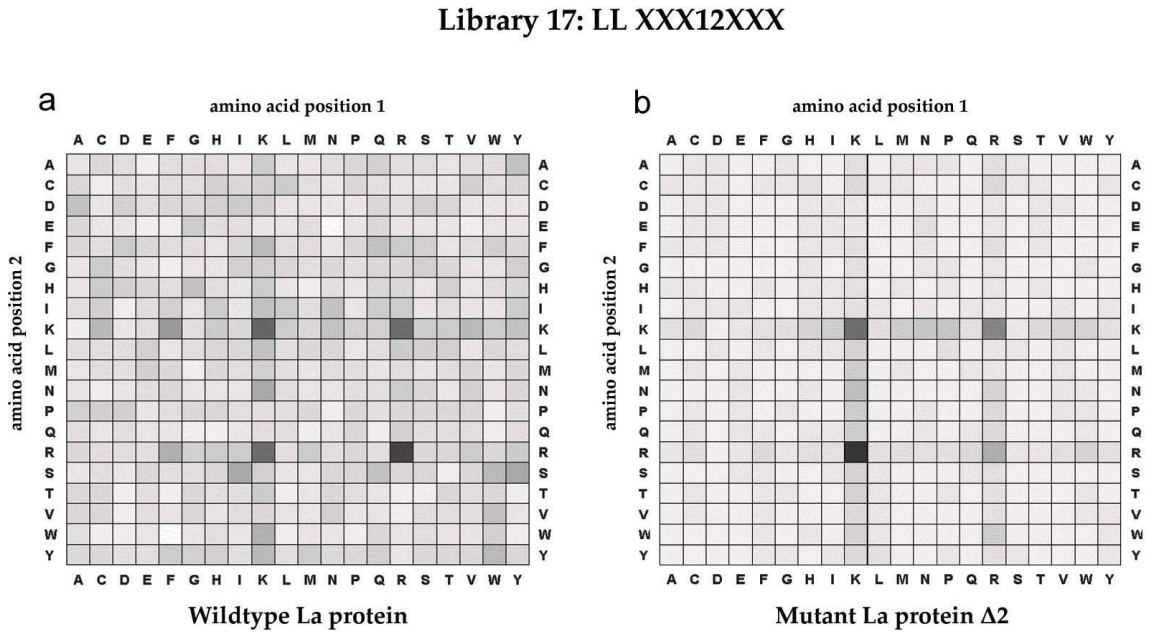

Figure 3-17. Comparison of intensities within the Library 17 XXX12XXX after binding assays
Comparison of intensities within the Library 17 XXX12XXX after binding assays with a) Mutant La protein and b) Wild type La protein using the Corel Chart software. Note that the wildtype La protein binds with a stronger affinity to "RR" spots and the mutant La protein Δ2 binds to "KK" spots with greater affinity.

These were then used to generate the next set of libraries in order to unravel the exact sequence of the binding peptides. The next library used was thus Lib 20 LL XX1RR2XX. The same procedure was followed as for the earlier assays. These resulted in a series of spots, as was expected.

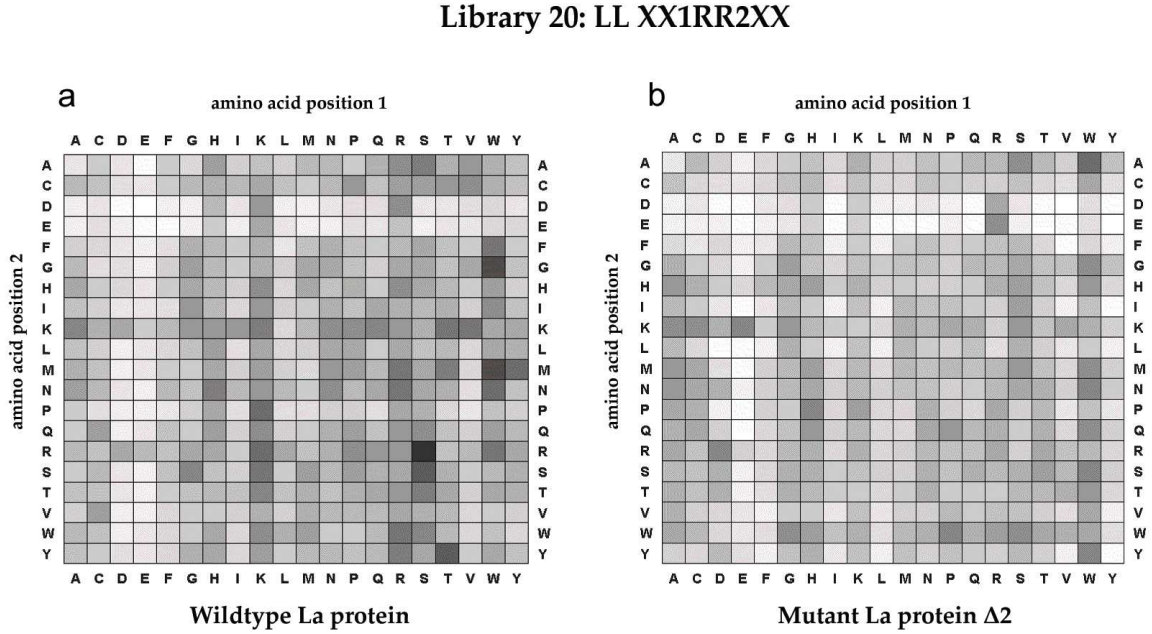

Figure 3-18. Comparison of intensities within the Library 20 LL XX1RR2XX
Comparison of intensities within the Library 20 LL XX1RR2XX after binding assays with a) Mutant La protein and b) Wild type La protein using the Corel Chart software. Note the spots such as "SR", "WM" and "WG" bound by the Wildtype La protein with higher intensity than the Mutant La protein Δ2. These are also plotted as a graph in the figure 3-19.

Based on Mutant and Wildtype La spot comparisons, 25 spots were selected as listed below to make the next library. These were selected based on the intensity of the spots as in the earlier case. In this case, too, the positive spots were the ones that showed a higher intensity of binding with the Wildtype La as compared to the Mutant La protein binding assays with the same library.

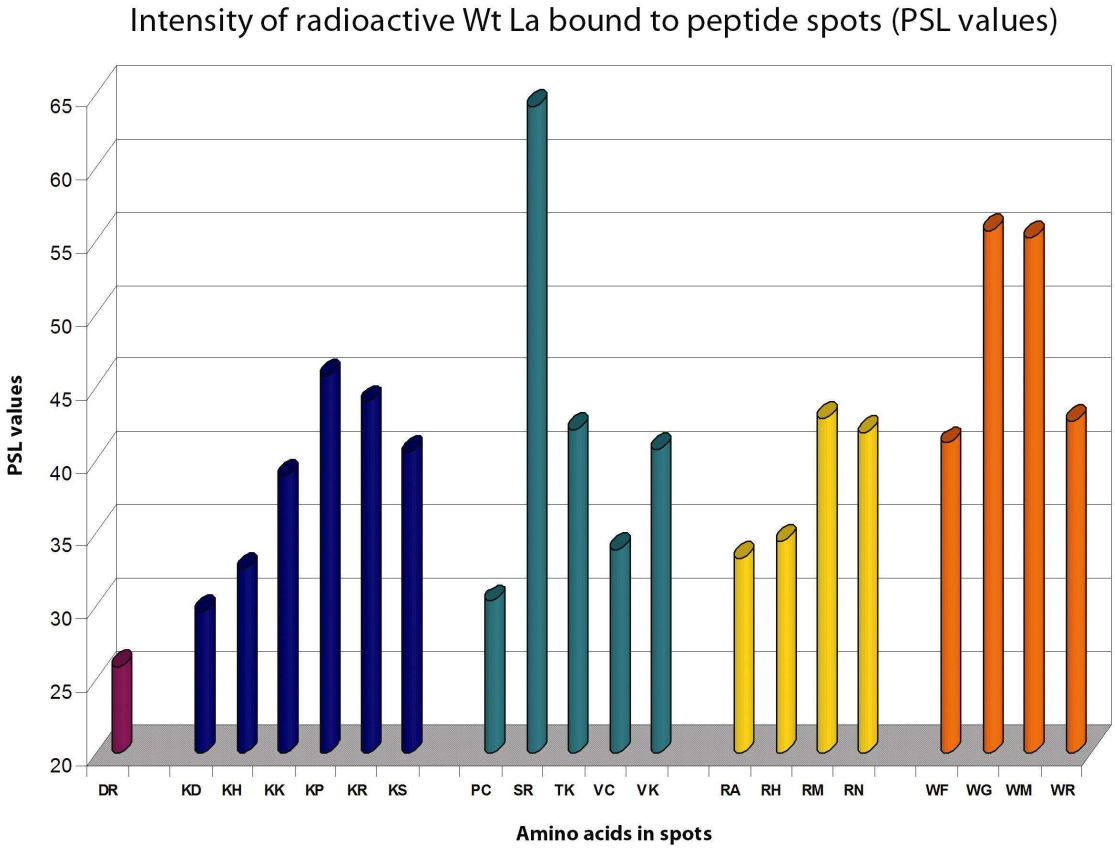

Figure 3-19. Comparison of spot intensities bound by Wildtype La protein to the Library 20 (PSL: Photo stimulated)

As can be seen in the figure 3-19, the peptides with the ''**S**RR**R**'' (serine, arginine) sequence give the highest signal. Also, peptides containing tryptophan (**W**) gave fairly strong positive results. Peptides containing lysine (**K**) also showed higher intensities. The 20 spots with the highest intensities were chosen as positive spots. In addition, 5 sequences were chosen which were in comparison, negative for the Wildtype La binding assay and at the same time positive for the Mutant La protein. These served as negative controls in the next set of libraries (figure 3-19).

Some trends were further reconfirmed in this set of binding assays. The peptides with the structure XX**K**RR**K**XX showed higher intensity of binding in the Mutant La binding assay as well which was in accordance with the results obtained from the Library 17 results, where the lysine sequences showed a comparatively stronger intensity in Mutant La binding assays. However the spots with lysine residues such as XX**V**RR**K**XX showed a comparatively higher signal for Wildtype La binding assays as can be seen in the Corel Chart representations below.

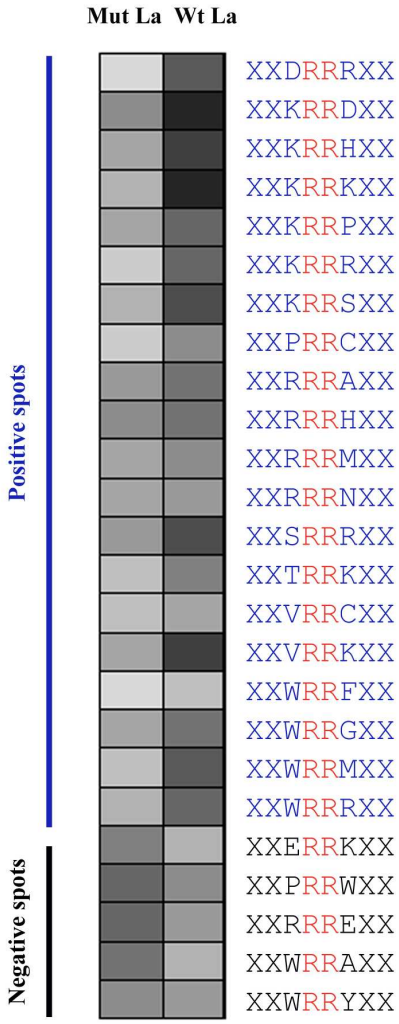

Figure 3-20. Binding assays with the positive spots chosen from the screening of the Library 20 LL XX1RR2XX

3.6.2 Binding assay with PNA membranes

These studies were carried out in an effort to identify PNA sequences that mimic the binding between the La protein with the Hepatitis B viral RNA. Peptide Nucleic Acid (PNA) libraries with 7 positions for variable nucleotides (a, t, g and c), from which the positions of 3 for any given spot were known were screened.

Starting libraries screened	Sequence	PNA residues in spots
Lib 21 (1)	PNA LC XBBBBBX	Mut spots similar to WT
Lib 21 (2)	PNA DC XBBBBBX	Mut spots similar to WT
Lib 21 (3)	PNA LL XBBBBBX	''gg'' spots
Lib 21 (4)	PNA DL XBBBBBX	Mut spots similar to WT

Table 3-3. PNA Libraries screened using binding assays with the Mutant and Wildtype La proteins

PNA libraries screened and their sequences are listed. Also defined positions of the identified spots that showed binding are also listed. B stands for any of the four PNA bases. For the position X; the amino acids can be **DL**:D aa linear, **LL**:L aa linear, **DC**:D aa cyclic, **LC**: L aa cyclic.

Again, as observed in the case of the Library 17, here too the libraries of PNA with linear sequences showed a positive lighting up of spots as compared to the Mutant La. The binding assays thus carried out, resulted in the lighting up of spots in the Wildtype La binding assays having two guanines in the sequence. However, spots with one or three guanines were found to show comparatively weak binding. Based on this, the next set of PNAs was synthesised.

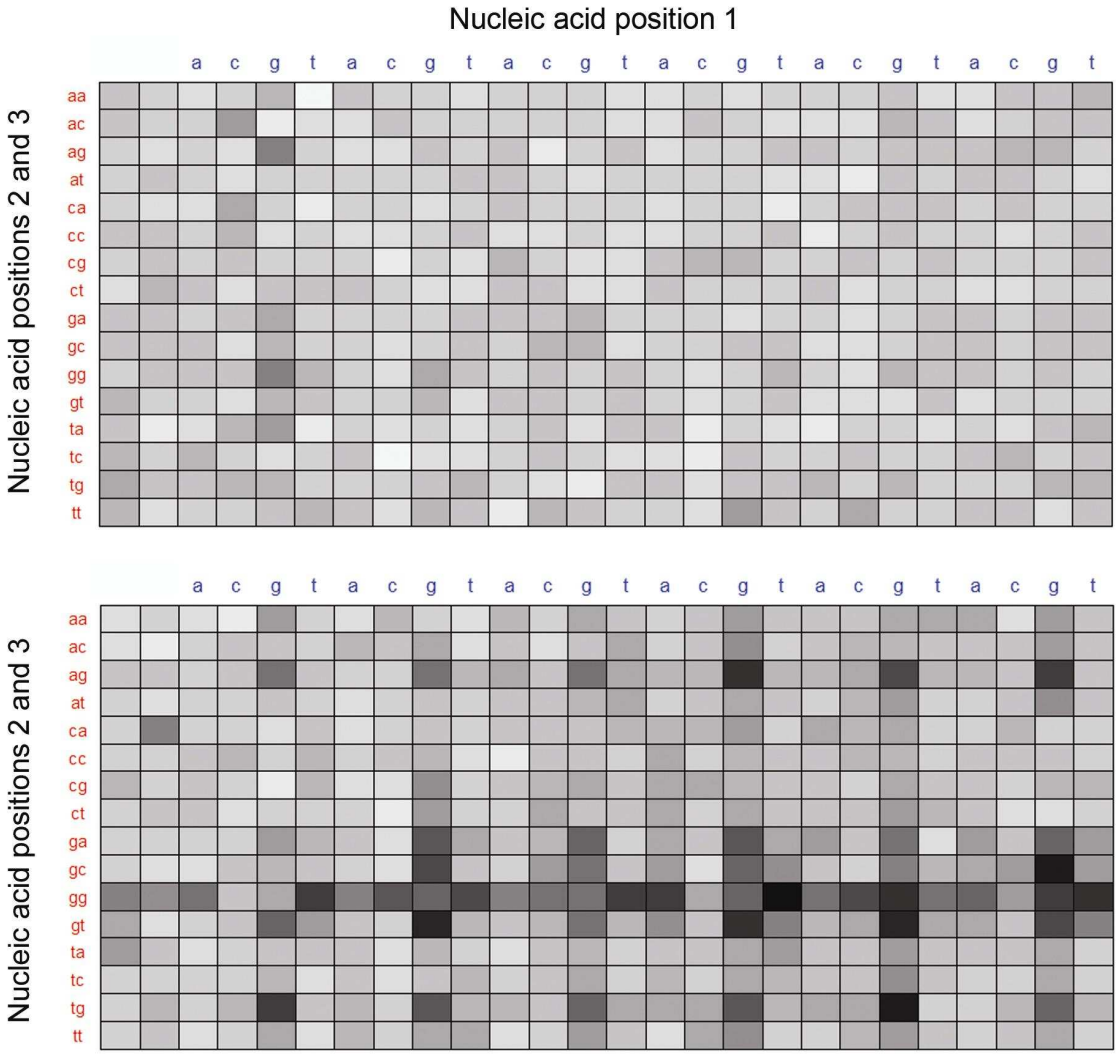

Figure 3-21. Comparison of intensities within the Library 21 PNA LL XBBBBBX.
Comparison of intensities within the Library 21 PNA LL XBBBBBX library after binding assays with a) mutant La protein and b) wildtype La protein using the Corel chart software.

In this case, 15 positive and 10 negative spots were chosen using similar criteria as were used for Library 17. The spot with the sequences XggBBcX and XtgBBgX showed comparatively higher signals. However, further assays were not possible with these sets of libraries since the Amersham's TNT kit used to make the radioactive La proteins failed to give the right molecular weight of the La protein or in some trials failed to make any at all. And hence, the binding assays were repeated with recombinant non-radioactive protein made *in vitro* in *E. coli* BL21.

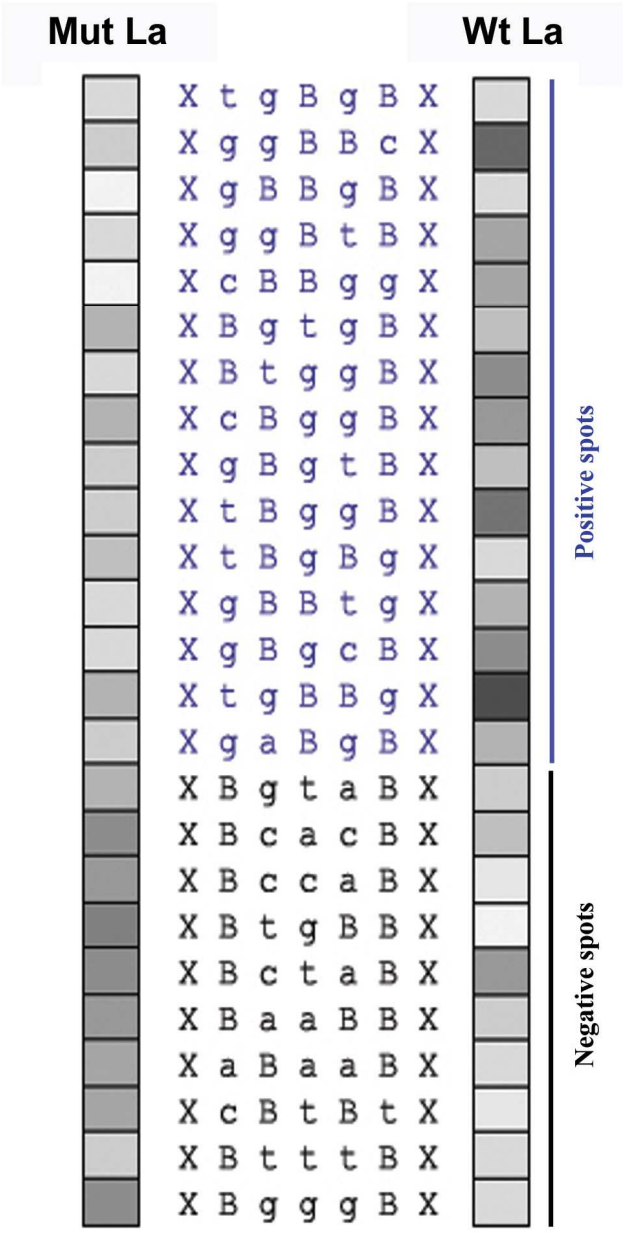

Figure 3-22. Binding Assays with the positive spots chosen from the screening of the Library 21 PNA LL XBBBBBX.

At this point in the project, the *in vitro* transcription & translation kit from Promega yielded radioactively labelelled Wildtype and mutant La proteins of several sizes. A similar *in vitro* transcription- translation kit from Ambion was also tried, but this also did not yield radioactively labeled proteins of the right size.

As an alternate, *E. coli* were transformed with wildtype and mutant La plasmids and the respective proteins were purified. Binding assays on SPOT libraries were repeated with the non-radioactive mutant and wildtype La proteins. Anti-La antibodies were used to detect

the La proteins bound to the cellulose membranes of SPOT libraries. The HRP-conjugated secondary antibodies used were detected using the enhanced chemiluminescence system from Amersham.

These assays differed from the radioactive protein assays because they involved several steps, which eventually were not as sensitive as radioactive detection of the protein peptide interactions and binding. The binding assays gave no spots with the libraries that had to be repeated (Lib 17 and Lib 21 PNA). And hence, no further peptide sequences could be synthesised and tried. A more sensitive alternative to ECL is needed to repeat these binding assays. Some leads to begin with could be ''RR'' spots from L amino acids linear octamer libraries or ''gg'' spots from PNA libraries since the earlier binding assays with radioactive proteins showed rather promising results.

4 Conclusions and Outlook

The main objective of this thesis was to develop a platform where cell based assays could be carried out on surfaces where chemical synthesis reactions were previously performed. This would enable the platform to be directly used for cell based assays once small molecule libraries are synthesised on it without the need to externally add the individual compounds to the cells.

The SPOT libraries used for screening in this study have several advantages. They can be screened in-situ and can be reused several times. The need to store large compound libraries which rapidly degrade over time is eliminated. There is no need to repetitively check compound integrity. Also they require only a small repertoire of chemical building blocks to synthesise them. The use of these libraries gave good results for identification of at least partial sequences of peptides that could bind to the wildtype La protein. The only limitation was of a sensitive detection system.

Such a platform was developed during the course of this thesis and tested for the growth of ten different cell lines commonly used for cell based assays. Cell growth was first analysed on polypropylene foils with simple chemical modifications and later fully synthesised acidic and basic peptides were also tested. The next step was to repeat known cellular phenotypes using this platform. 3Y1 cells grown on PP foils coated with poly L lysine showed the characteristic round morphology observed on lamellipodia induction. Also, cytokinesis was observed in PtK2 cell based assays using PP patches with DT-2 peptides synthesised on them. Thus, a polypropylene patch platform was established where synthesised small molecules resulted in observable phenotypes that could be documented using standard fluorescence microscopy procedures.

The next goal was to develop cell based assays using the model La protein that could detect the cleavage of the La protein when screened using small molecule libraries. A cygnet La vector was developed for this purpose by the group of Dr. Tilman Heise at the HPI in Hamburg. The cygnet La protein was tested in *in vitro* as well as *in vivo* assays. The *in vitro* results show that on enzymatic cleavage, the cygnet La protein loses its FRET signal which can be detected using spectrofluorimetry. Also, in the *in vivo* assays, cygnet La transfected

cells exhibit loss of the FRET signal on acceptor photobleaching. Thus it is now possible to use this cygnet La vector to screen small molecule libraries for compounds that can activate proteases that can cleave the La protein by screening for loss of the FRET signal in transfected cells. For an alternative strategy, a GFP NES La construct was also developed in Hamburg to facilitate screenings for translocation of the La protein from the nucleus to the cytoplasm in transfected A549 cells on cleavage by small molecule compounds. This was tested using a chemical compound, Epothilone which is a known apoptosis inducer in cells.

Also, recombinant radioactive wildtype and Mutant La proteins were used in binding assays with SPOT libraries to obtain peptide sequences that can bind to the Wildtype La protein and not to the Mutant La protein. The binding assays yielded partial sequences of peptides from LL XX1RR2XX libraries and PNA libraries with "gg" spots. It was not possible to obtain complete sequences since the *in vitro* transcription and translation kit did not yield the right size La proteins and repetition using other kits or non-radioactive proteins did not yield detectable results. However, the results obtained up to this stage were promising and should be further tested to obtain the complete sequences of these peptides. Such peptides could then be tested in cell based assays using the GFP NES La vector or the Cygnet La vector. Also, cells transfected with either of these two constructs can be used for carrying out cell based assays on PP foils synthesised with combinatorial libraries.

5 References

1. van Muijlwijk-Koezen, J. E., Timmerman, H., van der Sluis, R. P., van de Stolpe, A. C., Menge, W. M., Beukers, M. W., van der Graaf, P. H., de, G. M., & IJzerman, A. P. 2001, "Synthesis and use of FSCPX, an irreversible adenosine A1 antagonist, as a 'receptor knock-down' tool
Bioorg.Med.Chem.Lett., vol. 11, no. 6, pp. 815-818.

2. Xu, L., Chen, S., & Bergan, R. C. 2006, "MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer
Oncogene, vol. 25, no. 21, pp. 2987-2998.

3. Spring, D. R. 2005, "Chemical genetics to chemical genomics: small molecules offer big insights", *Chem.Soc.Rev.*, vol. 34, no. 6, pp. 472-482.

4. Allada, R., Emery, P., Takahashi, J. S., & Rosbash, M. 2001, "Stopping time: the genetics of fly and mouse circadian clocks", *Annu.Rev.Neurosci.*, vol. 24, pp. 1091-1119.

5. Emery, P. 2007, "Mutagenesis with Drosophila", *Methods Mol.Biol.*, vol. 362, pp. 187-195.

6. Bonafe, N. & Sellers, J. R. 1998, "Molecular characterization of myosin V from Drosophila melanogaster", *J.Muscle Res.Cell Motil.*, vol. 19, no. 2, pp. 129-141.

7. Stockwell, B. R. 2000, "Chemical genetics: ligand-based discovery of gene function", *Nat.Rev.Genet.*, vol. 1, no. 2, pp. 116-125.

8. Kim, E. E., Baker, C. T., Dwyer, M. D., Murcko, M. A., Rao, B. G., Tung, R. D., & Navia, M. A. 1995, "Crystal structure of HIV-1 protease in complex with VX-478, a potent and orally bioavailable inhibitor of the enzyme", *Journal of the American Chemical Society*, vol. 117, no. 3, pp. 1181-1182.

9. Brenner, C. 2004, "Chemical genomics in yeast", *Genome Biol.*, vol. 5, no. 9, p. 240.

10. Chan, T. F., Carvalho, J., Riles, L., & Zheng, X. F. 2000, "A chemical genomics approach toward understanding the global functions of the target of rapamycin protein (TOR)", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 24, pp. 13227-13232.

11. Stearns, T., Hoyt, M. A., & Botstein, D. 1990, "Yeast mutants sensitive to antimicrotubule drugs define three genes that affect microtubule function", *Genetics*, vol. 124, no. 2, pp. 251-262.

12. Crews, C. M. & Splittgerber, U. 1999, "Chemical genetics: exploring and controlling cellular processes with chemical probes", *Trends Biochem.Sci.*, vol. 24, no. 8, pp. 317-320.

13. van den Brink, G. R., Hardwick, J. C., Tytgat, G. N., Brink, M. A., Ten Kate, F. J., Van Deventer, S. J., & Peppelenbosch, M. P. 2001, "Sonic hedgehog regulates gastric gland morphogenesis in man and mouse", *Gastroenterology*, vol. 121, no. 2, pp. 317-328.

14. Chen, J. K., Taipale, J., Cooper, M. K., & Beachy, P. A. 2002, "Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened", *Genes Dev.*, vol. 16, no. 21, pp. 2743-2748.

15. Nanba, D., Nakanishi, Y., & Hieda, Y. 2003, "Role of Sonic hedgehog signaling in epithelial and mesenchymal development of hair follicles in an organ culture of embryonic mouse skin", *Dev.Growth Differ.*, vol. 45, no. 3, pp. 231-239.

16. Nissim, S., Allard, P., Bandyopadhyay, A., Harfe, B. D., & Tabin, C. J. 2007, "Characterization of a novel ectodermal signaling center regulating Tbx2 and Shh in the vertebrate limb", *Dev.Biol.*, vol. 304, no. 1, pp. 9-21.

17. Fenteany, G. & Schreiber, S. L. 1998, "Lactacystin, proteasome function, and cell fate", *J.Biol.Chem.*, vol. 273, no. 15, pp. 8545-8548.

18. Pasquini, L. A., Besio, M. M., Adamo, A. M., Pasquini, J. M., & Soto, E. F. 2000, "Lactacystin, a specific inhibitor of the proteasome, induces apoptosis and activates caspase-3 in cultured cerebellar granule cells", *J.Neurosci.Res.*, vol. 59, no. 5, pp. 601-611.

19. Fenteany, G., Standaert, R. F., Reichard, G. A., Corey, E. J., & Schreiber, S. L. 1994, "A beta-lactone related to lactacystin induces neurite outgrowth in a neuroblastoma cell line and inhibits cell cycle progression in an osteosarcoma cell line", *Proc.Natl.Acad.Sci.U.S.A*, vol. 91, no. 8, pp. 3358-3362.

20. Yoshida, M., Nishikawa, M., Nishi, K., Abe, K., Horinouchi, S., & Beppu, T. 1990, "Effects of leptomycin B on the cell cycle of fibroblasts and fission yeast cells", *Exp.Cell Res.*, vol. 187, no. 1, pp. 150-156.

21. Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S., & Beppu, T. 1994, "Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression", *J.Biol.Chem.*, vol. 269, no. 9, pp. 6320-6324.

22. Mahalingam, M. & Templeton, D. J. 1996, "Constitutive activation of S6 kinase by deletion of amino-terminal autoinhibitory and rapamycin sensitivity domains", *Mol.Cell Biol.*, vol. 16, no. 1, pp. 405-413.

23. Cardenas, M. E., Cutler, N. S., Lorenz, M. C., Di Como, C. J., & Heitman, J. 1999, "The TOR signaling cascade regulates gene expression in response to nutrients", *Genes Dev.*, vol. 13, no. 24, pp. 3271-3279.

24. Kay, J. E., Benzie, C. R., Goodier, M. R., Wick, C. J., & Doe, S. E. 1989, "Inhibition of T-lymphocyte activation by the immunosuppressive drug FK-506", *Immunology*, vol. 67, no. 4, pp. 473-477.

25. Reem, G. H., Shi, Y., & el Rouby, S. 1993, "FK-506 inhibits the IL-2-independent induction of the activation antigen CD 69", *Ann N.Y.Acad.Sci.*, vol. 685, pp. 52-54.

26. Hamel, E. 1996, "Antimitotic natural products and their interactions with tubulin", *Med.Res.Rev.*, vol. 16, no. 2, pp. 207-231.

27. Haggarty, S. J., Mayer, T. U., Miyamoto, D. T., Fathi, R., King, R. W., Mitchison, T. J., & Schreiber, S. L. 2000, "Dissecting cellular processes using small molecules: identification of colchicine-like, taxol-like and other small molecules that perturb mitosis",
Chem.Biol., vol. 7, no. 4, pp. 275-286.

28. Mayer, T. U., Kapoor, T. M., Haggarty, S. J., King, R. W., Schreiber, S. L., & Mitchison, T. J. 1999, "Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen",
Science, vol. 286, no. 5441, pp. 971-974.

29. Cox, C. D., Breslin, M. J., Mariano, B. J., Coleman, P. J., Buser, C. A., Walsh, E. S., Hamilton, K., Huber, H. E., Kohl, N. E., Torrent, M., Yan, Y., Kuo, L. C., & Hartman, G. D. 2005, "Kinesin spindle protein (KSP) inhibitors. Part 1: The discovery of 3,5-diaryl-4,5-dihydropyrazoles as potent and selective inhibitors of the mitotic kinesin KSP", *Bioorg.Med.Chem.Lett.*, vol. 15, no. 8, pp. 2041-2045.

30. Nakazawa, J., Yajima, J., Usui, T., Ueki, M., Takatsuki, A., Imoto, M., Toyoshima, Y. Y., & Osada, H. 2003, "A novel action of terpendole E on the motor activity of mitotic Kinesin Eg5", *Chem.Biol.*, vol. 10, no. 2, pp. 131-137.

31. Gartner, M., Sunder-Plassmann, N., Seiler, J., Utz, M., Vernos, I., Surrey, T., & Giannis, A. 2005, "Development and biological evaluation of potent and specific inhibitors of mitotic Kinesin Eg5", *Chembiochem.*, vol. 6, no. 7, pp. 1173-1177.

32. Nakai, R., Ishida, H., Asai, A., Ogawa, H., Yamamoto, Y., Kawasaki, H., Akinaga, S., Mizukami, T., & Yamashita, Y. 2006, "Telomerase inhibitors identified by a forward chemical genetics approach using a yeast strain with shortened telomere length", *Chem.Biol.*, vol. 13, no. 2, pp. 183-190.

33. Patel, V., Senderowicz, A. M., Pinto, D., Jr., Igishi, T., Raffeld, M., Quintanilla-Martinez, L., Ensley, J. F., Sausville, E. A., & Gutkind, J. S. 1998, "Flavopiridol, a novel cyclin-dependent kinase inhibitor, suppresses the growth of head and neck squamous cell carcinomas by inducing apoptosis", *J.Clin.Invest*, vol. 102, no. 9, pp. 1674-1681.

34. Mauro, M. J. & Druker, B. J. 2001, "STI571: targeting BCR-ABL as therapy for CML", *Oncologist.*, vol. 6, no. 3, pp. 233-238.

35. Lin, A. Y., Fisher, G. A., So, S., Tang, C., & Levitt, L. 2008, "Phase II study of imatinib in unresectable hepatocellular carcinoma", *Am.J.Clin.Oncol.*, vol. 31, no. 1, pp. 84-88.

36. Kelemen, B. R., Hsiao, K., & Goueli, S. A. 2002, "Selective in vivo inhibition of mitogen-activated protein kinase activation using cell permeable peptides", *J.Biol.Chem.*, vol. 277, no. 10, pp. 8741-8748.

37. Zewail, A., Xie, M. W., Xing, Y., Lin, L., Zhang, P. F., Zou, W., Saxe, J. P., & Huang, J. 2003, "Novel functions of the phosphatidylinositol metabolic pathway discovered by a chemical genomics screen with wortmannin", *Proc.Natl.Acad.Sci.U.S.A*, vol. 100, no. 6, pp. 3345-3350.

38. Ishizaki, T., Uehata, M., Tamechika, I., Keel, J., Nonomura, K., Maekawa, M., & Narumiya, S. 2000, "Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases", *Mol.Pharmacol.*, vol. 57, no. 5, pp. 976-983.

39. Ferreira, C. V., Justo, G. Z., Souza, A. C., Queiroz, K. C., Zambuzzi, W. F., Aoyama, H., & Peppelenbosch, M. P. 2006, "Natural compounds as a source of protein tyrosine phosphatase inhibitors: application to the rational design of small-molecule derivatives", *Biochimie*, vol. 88, no. 12, pp. 1859-1873.

40. Butler, M. S. 2008, "Natural products to drugs: natural product-derived compounds in clinical trials", *Nat.Prod.Rep.*, vol. 25, no. 3, pp. 475-516.

41. Butler, M. S. & Buss, A. D. 2006, "Natural products--the future scaffolds for novel antibiotics?", *Biochem.Pharmacol.*, vol. 71, no. 7, pp. 919-929.

42. Baker, D. D. & Alvi, K. A. 2004, "Small-molecule natural products: new structures, new activities", *Curr.Opin.Biotechnol.*, vol. 15, no. 6, pp. 576-583.

43. Jimeno, J. M. 2002, "A clinical armamentarium of marine-derived anti-cancer compounds", *Anticancer Drugs*, vol. 13 Suppl 1, p. S15-S19.

44. Mayer, A. M. & Gustafson, K. R. 2006, "Marine pharmacology in 2003-2004: anti-tumour and cytotoxic compounds", *Eur.J.Cancer*, vol. 42, no. 14, pp. 2241-2270.

45. Adrian, T. E. 2007, "Novel marine-derived anti-cancer agents", *Curr.Pharm.Des*, vol. 13, no. 33, pp. 3417-3426.

46. Haefner, B. 2003, "Drugs from the deep: marine natural products as drug candidates", *Drug Discov.Today*, vol. 8, no. 12, pp. 536-544.

47. Wessjohann, L. A. 2000, "Synthesis of natural-product-based compound libraries", *Curr.Opin.Chem.Biol.*, vol. 4, no. 3, pp. 303-309.

48. Gutierrez-Lugo, M. T. & Bewley, C. A. 2008, "Natural products, small molecules, and genetics in tuberculosis drug development", *J.Med.Chem.*, vol. 51, no. 9, pp. 2606-2612.

49. Fergus, S., Bender, A., & Spring, D. R. 2005, "Assessment of structural diversity in combinatorial synthesis", *Curr.Opin.Chem.Biol.*, vol. 9, no. 3, pp. 304-309.

50. Pelish, H. E., Westwood, N. J., Feng, Y., Kirchhausen, T., & Shair, M. D. 2001, "Use of biomimetic diversity-oriented synthesis to discover galanthamine-like molecules with biological properties beyond those of the natural product", *Journal of the American Chemical Society*, vol. 123, no. 27, pp. 6740-6741.

51. Merrifield, R. B. 1965, "SOLID-PHASE PEPTIDE SYNTHESES", *Endeavour*, vol. 24, pp. 3-7.

52. Mitchell, A. R. 2008, "Bruce Merrifield and solid-phase peptide synthesis: A historical assessment", *Biopolymers*, vol. 90, no. 3, pp. 175-184.

53. Geysen, H. M., Meloen, R. H., & Barteling, S. J. 1984, "Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid", *Proc.Natl.Acad.Sci.U.S.A*, vol. 81, no. 13, pp. 3998-4002.

54. Frank, R. 1995, "Simultaneous and combinatorial chemical synthesis techniques for the generation and screening of molecular diversity", *J.Biotechnol.*, vol. 41, no. 2-3, pp. 259-272.

55. Fodor, S. P., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T., & Solas, D. 1991, "Light-directed, spatially addressable parallel chemical synthesis", *Science*, vol. 251, no. 4995, pp. 767-773.

56. Furka, A., Sebestyen, F., Asgedom, M., & Dibo, G. 1991, "General method for rapid synthesis of multicomponent peptide mixtures", *Int.J.Pept.Protein Res.*, vol. 37, no. 6, pp. 487-493.

57. Houghten, R. A., Pinilla, C., Blondelle, S. E., Appel, J. R., Dooley, C. T., & Cuervo, J. H. 1991, "Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery",
Nature, vol. 354, no. 6348, pp. 84-86.

58. Colas, P., Cohen, B., Jessen, T., Grishina, I., McCoy, J., & Brent, R. 1996, "Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2", *Nature*, vol. 380, no. 6574, pp. 548-550.

59. Adey, N. B. & Kay, B. K. 1996, "Identification of calmodulin-binding peptide consensus sequences from a phage-displayed random peptide library", *Gene*, vol. 169, no. 1, pp. 133-134.

60. Yayon, A., Aviezer, D., Safran, M., Gross, J. L., Heldman, Y., Cabilly, S., Givol, D., & Katchalski-Katzir, E. 1993, "Isolation of peptides that inhibit binding of basic fibroblast growth factor to its receptor from a random phage-epitope library", *Proc.Natl.Acad.Sci.U.S.A*, vol. 90, no. 22, pp. 10643-10647.

61. Dolle, R. E. & Nelson, K. H., Jr. 1999, "Comprehensive survey of combinatorial library synthesis: 1998", *J.Comb.Chem.*, vol. 1, no. 4, pp. 235-282.

62. Sullivan, R. W., Bigam, C. G., Erdman, P. E., Palanki, M. S., Anderson, D. W., Goldman, M. E., Ransone, L. J., & Suto, M. J. 1998, "2-Chloro-4-(trifluoromethyl)pyrimidine-5-N-(3',5'- bis(trifluoromethyl)phenyl)-carboxamide: a potent inhibitor of NF-kappa B- and AP-1-mediated gene expression identified using solution-phase combinatorial chemistry", *J.Med.Chem.*, vol. 41, no. 4, pp. 413-419.

63. Mueller, O. J., Kaul, F., Weitzman, M. D., Pasqualini, R., Arap, W., Kleinschmidt, J. A., & Trepel, M. 2003, "Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors", *Nat.Biotechnol.*, vol. 21, no. 9, pp. 1040-1046.

64. Hajitou, A., Rangel, R., Trepel, M., Soghomonyan, S., Gelovani, J. G., Alauddin, M. M., Pasqualini, R., & Arap, W. 2007, "Design and construction of targeted AAVP vectors for mammalian cell transduction", *Nat.Protoc.*, vol. 2, no. 3, pp. 523-531.

65. Hill, H. R. & Stockley, P. G. 1996, "Phage presentation",
Mol.Microbiol., vol. 20, no. 4, pp. 685-692.

66. Kondo, A. & Ueda, M. 2004, "Yeast cell surface display--applications of molecular display", *Appl.Microbiol.Biotechnol.*, vol. 64, no. 1, pp. 28-40.

67. Linn, H., Ermekova, K. S., Rentschler, S., Sparks, A. B., Kay, B. K., & Sudol, M. 1997, "Using molecular repertoires to identify high-affinity peptide ligands of the WW domain of human and mouse YAP", *Biol.Chem.*, vol. 378, no. 6, pp. 531-537.

68. Vetter, S. W. & Zhang, Z. Y. 2002, "Probing the phosphopeptide specificities of protein tyrosine phosphatases, SH2 and PTB domains with combinatorial library methods", *Curr.Protein Pept.Sci.*, vol. 3, no. 4, pp. 365-397.

69. Luban, J. & Goff, S. P. 1995, "The yeast two-hybrid system for studying protein-protein interactions", *Curr.Opin.Biotechnol.*, vol. 6, no. 1, pp. 59-64.

70. Koegl, M. & Uetz, P. 2007, "Improving yeast two-hybrid screening systems", *Brief.Funct.Genomic.Proteomic.*, vol. 6, no. 4, pp. 302-312.

71. Lentze, N. & Auerbach, D. 2008, "The yeast two-hybrid system and its role in drug discovery", *Expert.Opin.Ther.Targets.*, vol. 12, no. 4, pp. 505-515.

72. Blackwell, H. E. 2006, "Hitting the SPOT: small-molecule macroarrays advance combinatorial synthesis", *Curr.Opin.Chem.Biol.*, vol. 10, no. 3, pp. 203-212.

73. Schena, M., Shalon, D., Davis, R. W., & Brown, P. O. 1995, "Quantitative monitoring of gene expression patterns with a complementary DNA microarray", *Science*, vol. 270, no. 5235, pp. 467-470.

74. Lockhart, D. J. & Winzeler, E. A. 2000, "Genomics, gene expression and DNA arrays", *Nature*, vol. 405, no. 6788, pp. 827-836.

75. Chiosis, G. & Brodsky, J. L. 2005, "Small molecule microarrays: from proteins to mammalian cells - are we there yet?", *Trends Biotechnol.*, vol. 23, no. 6, pp. 271-274.

76. Gosalia, D. N. & Diamond, S. L. 2003, "Printing chemical libraries on microarrays for fluid phase nanoliter reactions", *Proc.Natl.Acad.Sci.U.S.A*, vol. 100, no. 15, pp. 8721-8726.

77. Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., Bloomfield, C. D., & Lander,

E. S. 1999, "Molecular classification of cancer: class discovery and class prediction by gene expression monitoring", *Science*, vol. 286, no. 5439, pp. 531-537.

78. Perou, C. M., Sorlie, T., Eisen, M. B., van de, R. M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lonning, P. E., Borresen-Dale, A. L., Brown, P. O., & Botstein, D. 2000, "Molecular portraits of human breast tumours", *Nature*, vol. 406, no. 6797, pp. 747-752.

79. MacBeath, G. & Schreiber, S. L. 2000, "Printing Proteins as Microarrays for High-Throughput Function Determination", *Science*, vol. 289, no. 5485, pp. 1760-1763.

80. Zhu, H., Klemic, J. F., Chang, S., Bertone, P., Casamayor, A., Klemic, K. G., Smith, D., Gerstein, M., Reed, M. A., & Snyder, M. 2000, "Analysis of yeast protein kinases using protein chips", *Nat.Genet.*, vol. 26, no. 3, pp. 283-289.

81. Kuruvilla, F. G., Shamji, A. F., Sternson, S. M., Hergenrother, P. J., & Schreiber, S. L. 2002, "Dissecting glucose signalling with diversity-oriented synthesis and small-molecule microarrays", *Nature*, vol. 416, no. 6881, pp. 653-657.

82. Arenkov, P., Kukhtin, A., Gemmell, A., Voloshchuk, S., Chupeeva, V., & Mirzabekov, A. 2000, "Protein Microchips: Use for Immunoassay and Enzymatic Reactions", *Analytical Biochemistry*, vol. 278, no. 2, pp. 123-131.

83. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., & Snyder, M. 2001, "Global analysis of protein activities using proteome chips", *Science*, vol. 293, no. 5537, pp. 2101-2105.

84. Jhaveri, S., Rajendran, M., & Ellington, A. D. 2000, "In vitro selection of signaling aptamers", *Nat.Biotechnol.*, vol. 18, no. 12, pp. 1293-1297.

85. Mayer, G., Blind, M., Nagel, W., Bohm, T., Knorr, T., Jackson, C. L., Kolanus, W., & Famulok, M. 2001, "Controlling small guanine-nucleotide-exchange factor function through cytoplasmic RNA intramers", *Proc.Natl.Acad.Sci.U.S.A*, vol. 98, no. 9, pp. 4961-4965.

86. Famulok, M., Mayer, G., & Blind, M. 2000, "Nucleic acid aptamers-from selection in vitro to applications in vivo", *Acc.Chem.Res.*, vol. 33, no. 9, pp. 591-599.

87. Bruno, J. G. & Kiel, J. L. 1999, "In vitro selection of DNA aptamers to anthrax spores with electrochemiluminescence detection", *Biosens.Bioelectron.*, vol. 14, no. 5, pp. 457-464.

88. Kline, M., Donovan, K., Wellik, L., Lust, C., Jin, W., Moon-Tasson, L., Xiong, Y., Witzig, T. E., Kumar, S., Rajkumar, S. V., & Lust, J. A. 2007, "Cytokine and chemokine profiles in multiple myeloma; significance of stromal interaction and correlation of IL-8 production with disease progression", *Leuk.Res.*, vol. 31, no. 5, pp. 591-598.

89. Shafer, M. W., Mangold, L., Partin, A. W., & Haab, B. B. 2007, "Antibody array profiling reveals serum TSP-1 as a marker to distinguish benign from malignant prostatic disease", *Prostate*, vol. 67, no. 3, pp. 255-267.

90. Jiang, X., Roth, L., Han, S., & Li, X. 2008, "SH2 domain-based tyrosine phosphorylation array", *Methods Mol.Biol.*, vol. 441, pp. 153-161.

91. Kilic, A., Schuchert, M. J., Luketich, J. D., Landreneau, R. J., Lokshin, A. E., Bigbee, W. L., & El Hefnawy, T. 2008, "Use of novel autoantibody and cancer-related protein arrays for the detection of esophageal adenocarcinoma in serum", *J.Thorac.Cardiovasc.Surg.*, vol. 136, no. 1, pp. 199-204.

92. Eichler, J. 2005, "Synthetic peptide arrays and peptide combinatorial libraries for the exploration of protein-ligand interactions and the design of protein inhibitors", *Comb.Chem.High Throughput.Screen.*, vol. 8, no. 2, pp. 135-143.

93. Okochi, M., Hayashi, H., Ito, A., Kato, R., Tamura, Y., Sato, N., & Honda, H. 2008, "Identification of HLA-A24-restricted epitopes with high affinities to Hsp70 using peptide arrays", *J.Biosci.Bioeng.*, vol. 105, no. 3, pp. 198-203.

94. Beutling, U., Stading, K., Stradal, T., & Frank, R. 2008, "Large-scale analysis of protein-protein interactions using cellulose-bound peptide arrays", *Adv.Biochem.Eng Biotechnol.*, vol. 110, pp. 115-152.

95. Frank R. 1992. Spot synthesis: An easy technique for the positionally addressable, parallel chemical synthesis on a membrane support.Tetrahedron 48, 9217-9232.

96. Hashimoto, K. & Ishimori, Y. 2001, "Preliminary evaluation of electrochemical PNA array for detection of single base mismatch mutations", *Lab Chip.*, vol. 1, no. 1, pp. 61-63.

97. Germini, A., Rossi, S., Zanetti, A., Corradini, R., Fogher, C., & Marchelli, R. 2005, "Development of a peptide nucleic acid array platform for the detection of genetically modified organisms in food", *J.Agric.Food Chem.*, vol. 53, no. 10, pp. 3958-3962.

98. Kanter, J. L., Narayana, S., Ho, P. P., Catz, I., Warren, K. G., Sobel, R. A., Steinman, L., & Robinson, W. H. 2006, "Lipid microarrays identify key mediators of autoimmune brain inflammation", *Nat.Med.*, vol. 12, no. 1, pp. 138-143.

99. Lawrie, C. H., Marafioti, T., Hatton, C. S., Dirnhofer, S., Roncador, G., Went, P., Tzankov, A., Pileri, S. A., Pulford, K., & Banham, A. H. 2006, "Cancer-associated carbohydrate identification in Hodgkin's lymphoma by carbohydrate array profiling", *Int.J.Cancer*, vol. 118, no. 12, pp. 3161-3166.

100. Gyorgy, B., Tothfalusi, L., Nagy, G., Pasztoi, M., Geher, P., Lorincz, Z., Polgar, A., Rojkovich, B., Ujfalussy, I., Poor, G., Pocza, P., Wiener, Z., Misjak, P., Koncz, A., Falus, A., & Buzas, E. I. 2008, "Natural autoantibodies reactive with glycosaminoglycans in rheumatoid arthritis", Arthritis Res.Ther., vol. 10, no. 5, p. R110.

101. Gao, F. & Du, G. H. 2006, "Application of chemical arrays in screening elastase inhibitors", *Comb.Chem.High Throughput.Screen.*, vol. 9, no. 5, pp. 381-388.

102. Wu, J., Yan, F., Zhang, X., Yan, Y., Tang, J., & Ju, H. 2008, "Disposable reagentless electrochemical immunosensor array based on a biopolymer/sol-gel membrane for simultaneous measurement of several tumor markers", *Clin.Chem.*, vol. 54, no. 9, pp. 1481-1488.

103. Li, Y., Keller, D. M., Scott, J. D., & Lu, H. 2005, "CK2 phosphorylates SSRP1 and inhibits its DNA-binding activity", *J.Biol.Chem.*, vol. 280, no. 12, pp. 11869-11875.

104. Saxinger, C., Conrads, T. P., Goldstein, D. J., & Veenstra, T. D. 2005, "Fully automated synthesis of (phospho)peptide arrays in microtiter plate wells provides efficient access to protein tyrosine kinase characterization", *BMC.Immunol.*, vol. 6, no. 1, p. 1.

105. Broude, N. E., Woodward, K., Cavallo, R., Cantor, C. R., & Englert, D. 2001, "DNA microarrays with stem-loop DNA probes: preparation and applications", *Nucleic Acids Res.*, vol. 29, no. 19, p. E92.

106. Dahl, A., Sultan, M., Jung, A., Schwartz, R., Lange, M., Steinwand, M., Livak, K. J., Lehrach, H., & Nyarsik, L. 2007, "Quantitative PCR based expression analysis on a nanoliter scale using polymer nano-well chips", *Biomed.Microdevices.*, vol. 9, no. 3, pp. 307-314.

107. Dikmans, A. J., Morr, M., Zander, N., Adler, F., Turk, G., & Frank, R. 2004, "A new compact disc format of high density array synthesis applied to peptide nucleic acids and in situ MALDI analysis", *Mol.Divers.*, vol. 8, no. 3, pp. 197-207.

108. Fang, Y., Frutos, A. G., & Lahiri, J. 2002, "G-protein-coupled receptor microarrays", *Chembiochem.*, vol. 3, no. 10, pp. 987-991.

109. Li, S., Marthandan, N., Bowerman, D., Garner, H. R., & Kodadek, T. 2005, "Photolithographic synthesis of cyclic peptide arrays using a differential deprotection strategy", *Chem.Commun.(Camb.)* no. 5, pp. 581-583.

110. Matson, R. S., Milton, R. C., Cress, M. C., Chan, T. S., & Rampal, J. B. 2007, "Printing low density protein arrays in microplates", *Methods Mol.Biol.*, vol. 381, pp. 339-361.

111. Melkko, S., Dumelin, C. E., Scheuermann, J., & Neri, D. 2007, "Lead discovery by DNA-encoded chemical libraries", *Drug Discov.Today*, vol. 12, no. 11-12, pp. 465-471.

112. Ramachandran, N., Hainsworth, E., Bhullar, B., Eisenstein, S., Rosen, B., Lau, A. Y., Walter, J. C., & LaBaer, J. 2004, "Self-assembling protein microarrays", *Science*, vol. 305, no. 5680, pp. 86-90.

113. Uttamchandani, M., Walsh, D. P., Yao, S. Q., & Chang, Y. T. 2005, "Small molecule microarrays: recent advances and applications", *Curr.Opin.Chem.Biol.*, vol. 9, no. 1, pp. 4-13.

114. Gonda, K. & Kikyo, N. 2006, "Nuclear remodeling assay in Xenopus egg extract", *Methods Mol.Biol.*, vol. 348, pp. 247-258.

115. Gilbert, A., Paccaud, J. P., & Carpentier, J. L. 1997, "Direct measurement of clathrin-coated vesicle formation using a cell free assay", *J.Cell Sci.*, vol. 110 (Pt 24), pp. 3105-3115.

116. Dayton, B. D., Chiou, W. J., Opgenorth, T. J., & Wu-Wong, J. R. 2000, "Direct determination of endothelin receptor antagonist levels in plasma using a scintillation proximity assay", *Life Sci.*, vol. 66, no. 10, pp. 937-945.

117. Jenh, C. H., Zhang, M., Wiekowski, M., Tan, J. C., Fan, X. D., Hegde, V., Patel, M., Bryant, R., Narula, S. K., Zavodny, P. J., & Chou, C. C. 1998, "Development of a CD28 receptor binding-based screen and identification of a biologically active inhibitor", *Anal.Biochem.*, vol. 256, no. 1, pp. 47-55.

118. Trivedi, V., Zhang, S. C., Stockinger, W., & Nohturfft, A. 2007, "A cell free scintillation proximity assay for studies on lysosome-to-phagosome targeting", *Sci.STKE.*, vol. 2007, no. 392, p. l3.

119. Weinreb, P. H., Yang, W. J., Violette, S. M., Couture, M., Kimball, K., Pepinsky, R. B., Lobb, R. R., & Josiah, S. 2002, "A cell free electrochemiluminescence assay for measuring beta1-integrin-ligand interactions", *Anal.Biochem.*, vol. 306, no. 2, pp. 305-313.

120. Overall, M. L., Marzuki, S., & Hertzog, P. J. 1989, "Comparison of different ELISAs for the detection of monoclonal antibodies to human interferon-alpha. Implications for antibody screening",
J.Immunol.Methods, vol. 119, no. 1, pp. 27-33.

121. Sarubbi, E., Yanofsky, S. D., Barrett, R. W., & Denaro, M. 1996, "A cell free, nonisotopic, high-throughput assay for inhibitors of type-I interleukin-1 receptor",
Anal.Biochem., vol. 237, no. 1, pp. 70-75.

122. Andrade, G., Barbosa-Stancioli, E. F., Piscitelli Mansur, A. A., Vasconcelos, W. L., & Mansur, H. S. 2006, "Design of novel hybrid organic-inorganic nanostructured biomaterials for immunoassay applications",
Biomed.Mater., vol. 1, no. 4, pp. 221-234.

123. Usuda, S., Okamoto, H., Tsuda, F., Tanaka, T., Miyakawa, Y., & Mayumi, M. 1998, "An enzyme-linked immunosorbent assay with monoclonal antibodies for the determination of phosphorylated hepatitis B core protein (p21c) in serum",
J.Virol.Methods, vol. 72, no. 1, pp. 95-103.

124. Hale, M. L. 2001, "Microtiter-based assay for evaluating the biological activity of ribosome-inactivating proteins",
Pharmacol.Toxicol., vol. 88, no. 5, pp. 255-260.

125. Jones, J. W., Greene, T. A., Grygon, C. A., Doranz, B. J., & Brown, M. P. 2008, "Cell free assay of G-protein-coupled receptors using fluorescence polarization",
J.Biomol.Screen., vol. 13, no. 5, pp. 424-429.

126. Shao, Y., Wu, Y., Chan, C. Y., McDonough, K., & Ding, Y. 2006, "Rational design and rapid screening of antisense oligonucleotides for prokaryotic gene modulation",
Nucleic Acids Res., vol. 34, no. 19, pp. 5660-5669.

127. Kawaguchi, T., Chen, Y. P., Norman, R. S., & Decho, A. W. 2008, "Rapid screening of quorum-sensing signal N-acyl homoserine lactones by an in vitro cell free assay",
Appl.Environ.Microbiol., vol. 74, no. 12, pp. 3667-3671.

128. Evans, D., Johnson, S., Laurenson, S., Davies, A. G., Ko, F. P., & Walti, C. 2008, "Electrical protein detection in cell lysates using high-density peptide-aptamer microarrays",
J.Biol., vol. 7, no. 1, p. 3.

129. Bhushan, B. Ed. 2007, *Springer Handbook of Nanotechnology*, 2nd rev. and extended ed., 2007, XLIV, 1916 p. 1593 illus. in color. With CD-ROM., Hardcover edn.

130. Franke, L., Schwarz, O., Muller-Kuhrt, L., Hoernig, C., Fischer, L., George, S., Tanrikulu, Y., Schneider, P., Werz, O., Steinhilber, D., & Schneider, G. 2007, "Identification of natural-product-derived inhibitors of 5-lipoxygenase activity by ligand-based virtual screening", *J.Med.Chem.*, vol. 50, no. 11, pp. 2640-2646.

131. Landro, J. A., Taylor, I. C., Stirtan, W. G., Osterman, D. G., Kristie, J., Hunnicutt, E. J., Rae, P. M., & Sweetnam, P. M. 2000, "HTS in the new millennium: the role of pharmacology and flexibility", *J.Pharmacol.Toxicol.Methods*, vol. 44, no. 1, pp. 273-289.

132. Oh, M., Kim, S. Y., Oh, Y. S., Choi, D. Y., Sin, H. J., Jung, I. M., & Park, W. J. 2003, "Cell based assay for beta-secretase activity", *Anal.Biochem.*, vol. 323, no. 1, pp. 7-11.

133. Hu, M., Schurdak, M. E., Puttfarcken, P. S., El Kouhen, R., Gopalakrishnan, M., & Li, J. 2007, "High content screen microscopy analysis of A beta 1-42-induced neurite outgrowth reduction in rat primary cortical neurons: neuroprotective effects of alpha 7 neuronal nicotinic acetylcholine receptor ligands", *Brain Res.*, vol. 1151, pp. 227-235.

134. Quelever, G., Kachidian, P., Melon, C., Garino, C., Laras, Y., Pietrancosta, N., Sheha, M., & Louis, K. J. 2005, "Enhanced delivery of gamma-secretase inhibitor DAPT into the brain via an ascorbic acid mediated strategy", *Org.Biomol.Chem.*, vol. 3, no. 13, pp. 2450-2457.

135. Chung, K. S., Ahn, J., Choi, C. H., Yim, N. H., Kang, C. M., Kim, C. H., Lee, K., Park, H. M., Song, K. B., & Won, M. 2008, "Fission Yeast-Based Screening to Identify Putative HDAC Inhibitors using a Telomeric Reporter Strain", *Mol.Cells*, vol. 26, no. 1.

136. Krejci, P., Pejchalova, K., & Wilcox, W. R. 2007, "Simple, mammalian cell based assay for identification of inhibitors of the Erk MAP kinase pathway", *Invest New Drugs*, vol. 25, no. 4, pp. 391-395.

137. Blasi, M. F., Ventura, I., Aquilina, G., Degan, P., Bertario, L., Bassi, C., Radice, P., & Bignami, M. 2006, "A human cell based assay to evaluate the effects of alterations in the MLH1 mismatch repair gene", *Cancer Res.*, vol. 66, no. 18, pp. 9036-9044.

138. Raemy-Schenk, A. M., Trouble, S., Gaillard, P., Page, P., Gotteland, J. P., Scheer, A., Lang, P., & Yeow, K. 2006, "A cellular assay for measuring the modulation of glucose production in H4IIE cells", *Assay.Drug Dev.Technol.*, vol. 4, no. 5, pp. 525-533.

139. Yeow, K., Novo-Perez, L., Gaillard, P., Page, P., Gotteland, J. P., Scheer, A., & Lang, P. 2006, "A cellular assay for measuring the inhibition of glycogen synthase kinase-3 via the accumulation of beta-catenin in Chinese hamster ovary clone K1 cells", *Assay.Drug Dev.Technol.*, vol. 4, no. 4, pp. 451-460.

140. Skorey, K., Waddleton, D., Therien, M., & Leriche, T. 2006, "Enzyme occupancy measurement of intracellular protein tyrosine phosphatase 1B using photoaffinity probes", *Anal.Biochem.*, vol. 349, no. 1, pp. 49-61.

141. Bulmer, A. C., Blanchfield, J. T., Coombes, J. S., & Toth, I. 2008, "In vitro permeability and metabolic stability of bile pigments and the effects of hydrophilic and lipophilic modification of biliverdin", *Bioorg.Med.Chem.*, vol. 16, no. 7, pp. 3616-3625.

142. Wu, L., Feng, B. S., He, S. H., Zheng, P. Y., Croitoru, K., & Yang, P. C. 2007, "Bacterial peptidoglycan breaks down intestinal tolerance via mast cell activation: the role of TLR2 and NOD2", *Immunol.Cell Biol.*, vol. 85, no. 7, pp. 538-545.

143. Wu, C. Y., Jan, J. T., Ma, S. H., Kuo, C. J., Juan, H. F., Cheng, Y. S., Hsu, H. H., Huang, H. C., Wu, D., Brik, A., Liang, F. S., Liu, R. S., Fang, J. M., Chen, S. T., Liang, P. H., & Wong, C. H. 2004, "Small molecules targeting severe acute respiratory syndrome human coronavirus", *Proc.Natl.Acad.Sci.U.S.A*, vol. 101, no. 27, pp. 10012-10017.

144. Ladner, S. K., Otto, M. J., Barker, C. S., Zaifert, K., Wang, G. H., Guo, J. T., Seeger, C., & King, R. W. 1997, "Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication", *Antimicrob.Agents Chemother.*, vol. 41, no. 8, pp. 1715-1720.

145. Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J. C., Trent, J. M., Staudt, L. M., Hudson, J., Jr., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., & Brown, P. O. 1999, "The transcriptional program in the response of human fibroblasts to serum", *Science*, vol. 283, no. 5398, pp. 83-87.

146. Ruoslahti, E. 1997, "Stretching is good for a cell", *Science*, vol. 276, no. 5317, pp. 1345-1346.

147. Gumbiner, B. M. 1996, "Cell adhesion: the molecular basis of tissue architecture and morphogenesis", *Cell*, vol. 84, no. 3, pp. 345-357.

148. Singhvi, R., Kumar, A., Lopez, G. P., Stephanopoulos, G. N., Wang, D. I., Whitesides, G. M., & Ingber, D. E. 1994, "Engineering cell shape and function", *Science*, vol. 264, no. 5159, pp. 696-698.

149. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., & Ingber, D. E. 1997, "Geometric control of cell life and death", *Science*, vol. 276, no. 5317, pp. 1425-1428.

150. Nelson, C. M. & Chen, C. S. 2002, "Cell cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal", *FEBS Lett.*, vol. 514, no. 2-3, pp. 238-242.

151. Anderson, D. G., Levenberg, S., & Langer, R. 2004, "Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells", *Nat.Biotechnol.*, vol. 22, no. 7, pp. 863-866.

152. Bailey, S. N., Sabatini, D. M., & Stockwell, B. R. 2004, "Microarrays of small molecules embedded in biodegradable polymers for use in mammalian cell based screens", *Proc.Natl.Acad.Sci.U.S.A*, vol. 101, no. 46, pp. 16144-16149.

153. Bertrand, L., Parent, S., Caron, M., Legault, M., Joly, E., Angers, S., Bouvier, M., Brown, M., Houle, B., & Menard, L. 2002, "The BRET2/arrestin assay in stable recombinant cells: a platform to screen for compounds that interact with G protein-coupled receptors (GPCRS)", *J.Recept.Signal.Transduct.Res.*, vol. 22, no. 1-4, pp. 533-541.

154. Stockwell, B. R., Haggarty, S. J., & Schreiber, S. L. 1999, "High-throughput screening of small molecules in miniaturized mammalian cell based assays involving post-translational modifications", *Chem.Biol.*, vol. 6, no. 2, pp. 71-83.

155. Cooper, E. C. & Jan, L. Y. 1999, "Ion channel genes and human neurological disease: recent progress, prospects, and challenges", *Proc.Natl.Acad.Sci.U.S.A*, vol. 96, no. 9, pp. 4759-4766.

156. Tammela, P. & Vuorela, P. 2004, "Miniaturisation and validation of a cell based assay for screening of Ca2+ channel modulators", *J.Biochem.Biophys.Methods*, vol. 59, no. 3, pp. 229-239.

157. Slack, M., Kirchhoff, C., Moller, C., Winkler, D., & Netzer, R. 2006, "Identification of novel Kv1.3 blockers using a fluorescent cell based ion channel assay", *J.Biomol.Screen.*, vol. 11, no. 1, pp. 57-64.

158. Sun, Y., Firestein, G., Boyle, D., Gruber, H., & Cheung, H. 2007, "Longterm culture of telomerase-transduced rheumatoid arthritis fibroblast-like synoviocytes display a distinct gene expression pattern", *J.Rheumatol.*, vol. 34, no. 10, pp. 1959-1970.

159. Yoshizato, K., Shirotani, T., Furukawa, N., Taguchi, T., Motoshima, H., Toyonaga, T., Hirashima, Y., Kawashima, J., Ebina, Y., Shichiri, M., & Araki, E. 2001, "Identification of a cis-acting element and a novel trans-acting factor of the human insulin receptor gene in HepG2 and rat liver cells", *Biochem.Biophys.Res.Commun.*, vol. 280, no. 2, pp. 428-434.

160. Betrabet, S. S., Choudhuri, J., & Gill-Sharma, M. 2004, "Use of viral promoters in mammalian cell based bioassays: How reliable?", *J.Transl.Med.*, vol. 2, no. 1, p. 1.

161. Fung, P., Peng, K., Kobel, P., Dotimas, H., Kauffman, L., Olson, K., & Eglen, R. M. 2006, "A homogeneous cell based assay to measure nuclear translocation using beta-galactosidase enzyme fragment complementation",
Assay.Drug Dev.Technol., vol. 4, no. 3, pp. 263-272.

162. Huang, Y. Y., Shih, J. W., & Lin, J. J. 2007, "Establishing cell based reporter systems for the analysis of hTERT expression",
Methods Mol.Biol., vol. 405, pp. 87-96.

163. Sista, P., Edmiston, S., Darges, J. W., Robinson, S., & Burns, D. J. 1994, "A cell based reporter assay for the identification of protein kinase C activators and inhibitors"
Mol.Cell Biochem., vol. 141, no. 2, pp. 129-134.

164. Ji, D. B., Zhu, H. B., Ye, J., & Li, C. L. 2008, "Establishment of a cell based assay to screen regulators of the hypoxia-inducible factor-1-dependent vascular endothelial growth factor promoter",
Biol.Pharm.Bull., vol. 31, no. 12, pp. 2255-2259.

165. Hojo, H., Igawa, K., Ohba, S., Yano, F., Nakajima, K., Komiyama, Y., Ikeda, T., Lichtler, A. C., Woo, J. T., Yonezawa, T., Takato, T., & Chung, U. I. 2008, "Development of high-throughput screening system for osteogenic drugs using a cell based sensor",
Biochem.Biophys.Res.Commun., vol. 376, no. 2, pp. 375-379.

166. SHIMOMURA, O., JOHNSON, F. H., & SAIGA, Y. 1962, "Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea", *J.Cell Comp Physiol*, vol. 59, pp. 223-239.

167. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., & Cormier, M. J. 1992, "Primary structure of the Aequorea victoria green-fluorescent protein", *Gene*, vol. 111, no. 2, pp. 229-233.

168. Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., & Remington, S. J. 1996, "Crystal structure of the Aequorea victoria green fluorescent protein", *Science*, vol. 273, no. 5280, pp. 1392-1395.

169. Yang, F., Moss, L. G., & Phillips, G. N., Jr. 1996, "The molecular structure of green fluorescent protein", *Nat.Biotechnol.*, vol. 14, no. 10, pp. 1246-1251.

170. Zhang, J., Campbell, R. E., Ting, A. Y., & Tsien, R. Y. 2002, "Creating new fluorescent probes for cell biology", *Nat.Rev.Mol.Cell Biol.*, vol. 3, no. 12, pp. 906-918.

171. Pope, A. J., Haupts, U. M., & Moore, K. J. 1999, "Homogeneous fluorescence readouts for miniaturized high-throughput screening: theory and practice", *Drug Discov.Today*, vol. 4, no. 8, pp. 350-362.

172. Haupts, U., Rudiger, M., Ashman, S., Turconi, S., Bingham, R., Wharton, C., Hutchinson, J., Carey, C., Moore, K. J., & Pope, A. J. 2003, "Single-molecule detection technologies in miniaturized high-throughput screening: fluorescence intensity distribution analysis", *J.Biomol.Screen.*, vol. 8, no. 1, pp. 19-33.

173. Moore, K. J., Turconi, S., Ashman, S., Ruediger, M., Haupts, U., Emerick, V., V, & Pope, A. J. 1999, "Single Molecule Detection Technologies in Miniaturized High Throughput Screening: Fluorescence Correlation Spectroscopy", *J.Biomol.Screen.*, vol. 4, no. 6, pp. 335-354.

174. Prystay, L., Gagne, A., Kasila, P., Yeh, L. A., & Banks, P. 2001, "Homogeneous cell based fluorescence polarization assay for the direct detection of cAMP", *J.Biomol.Screen.*, vol. 6, no. 2, pp. 75-82.

175. Du, M. X., Sim, J., Fang, L., Yin, Z., Koh, S., Stratton, J., Pons, J., Wang, J. J., & Carte, B. 2004, "Identification of novel small-molecule inhibitors for human transketolase by high-throughput screening with fluorescent intensity (FLINT) assay", *J.Biomol.Screen.*, vol. 9, no. 5, pp. 427-433.

176. Marine, S., Zamiara, E., Smith, S. T., Stec, E. M., McGarvey, J., Kornienko, O., Jiang, G., Wong, K. K., Stack, J. H., Zhang, B. B., Ferrer, M., & Strulovici, B. 2006, "A miniaturized cell based fluorescence resonance energy transfer assay for insulin-receptor activation", *Anal.Biochem.*, vol. 355, no. 2, pp. 267-277.

177. Ruocco, K. M., Goncharova, E. I., Young, M. R., Colburn, N. H., McMahon, J. B., & Henrich, C. J. 2007, "A high-throughput cell based assay to identify specific inhibitors of transcription factor AP-1", *J.Biomol.Screen.*, vol. 12, no. 1, pp. 133-139.

178. Hwang, Y. C., Chu, J. J., Yang, P. L., Chen, W., & Yates, M. V. 2008, "Rapid identification of inhibitors that interfere with poliovirus replication using a cell based assay", *Antiviral Res.*, vol. 77, no. 3, pp. 232-236.

179. Giese, A., Bieschke, J., Eigen, M., & Kretzschmar, H. A. 2000, "Putting prions into focus: application of single molecule detection to the diagnosis of prion diseases", *Arch.Virol.Suppl* no. 16, pp. 161-171.

180. Parsons, M., Messent, A. J., Humphries, J. D., Deakin, N. O., & Humphries, M. J. 2008, "Quantification of integrin receptor agonism by fluorescence lifetime imaging", *J.Cell Sci.*, vol. 121, no. Pt 3, pp. 265-271.

181. Nyborg, A. C., Herl, L., Berezovska, O., Thomas, A. V., Ladd, T. B., Jansen, K., Hyman, B. T., & Golde, T. E. 2006, "Signal peptide peptidase (SPP) dimer formation as assessed by fluorescence lifetime imaging microscopy (FLIM) in intact cells", *Mol.Neurodegener.*, vol. 1, p. 16.

182. Albrecht, H., Zbinden, P., Rizzi, A., Villetti, G., Riccardi, B., Puccini, P., Catinella, S., & Imbimbo, B. P. 2004, "High throughput screening of beta-amyloid secretion inhibitors using homogenous time-resolved fluorescence", *Comb.Chem.High Throughput.Screen.*, vol. 7, no. 8, pp. 745-756.

183. Trinh, L., Ziegler, R., Watling, D., Snider, R. M., & Croze, E. 1999, "Development of an Immunofluorometric, High-Capacity, Cell Based Assay for the Measurement of Human Type I and Type II Interferons", *J.Biomol.Screen.*, vol. 4, no. 1, pp. 33-37.

184. Kobitski, A. Y., Nierth, A., Helm, M., Jaschke, A., & Nienhaus, G. U. 2007, "Mg2+-dependent folding of a Diels-Alderase ribozyme probed by single-molecule FRET analysis", *Nucleic Acids Res.*, vol. 35, no. 6, pp. 2047-2059.

185. Nettels, D., Hoffmann, A., & Schuler, B. 2008, "Unfolded protein and peptide dynamics investigated with single-molecule FRET and correlation spectroscopy from picoseconds to seconds", *J.Phys.Chem.B*, vol. 112, no. 19, pp. 6137-6146.

186. Birkmann, E., Henke, F., Funke, S. A., Bannach, O., Riesner, D., & Willbold, D. 2008, "A highly sensitive diagnostic assay for aggregate-related diseases, including prion diseases and Alzheimer's disease", *Rejuvenation.Res.*, vol. 11, no. 2, pp. 359-363.

187. Wolcke, J., Hunt, N., Jungmann, J., & Ullmann, D. 2006, "Early identification of false positives in high-throughput screening for activators of p53-DNA interaction", *J.Biomol.Screen.*, vol. 11, no. 4, pp. 341-350.

188. Girke, T., Cheng, L. C., & Raikhel, N. 2005, "ChemMine. A compound mining database for chemical genomics", *Plant Physiol*, vol. 138, no. 2, pp. 573-577.

189. Girke, T., Lauricha, J., Tran, H., Keegstra, K., & Raikhel, N. 2004, "The Cell Wall Navigator database. A systems-based approach to organism-unrestricted mining of protein families involved in cell wall metabolism", *Plant Physiol*, vol. 136, no. 2, pp. 3003-3008.

190. McCarthy, F. M., Bridges, S. M., Wang, N., Magee, G. B., Williams, W. P., Luthe, D. S., & Burgess, S. C. 2007, "AgBase: a unified resource for functional analysis in agriculture", *Nucleic Acids Res.*, vol. 35, no. Database issue, p. D599-D603.

191. Li, H., Gao, Z., Kang, L., Zhang, H., Yang, K., Yu, K., Luo, X., Zhu, W., Chen, K., Shen, J., Wang, X., & Jiang, H. 2006, "TarFisDock: a web server for identifying drug

targets with docking approach", *Nucleic Acids Res.*, vol. 34, no. Web Server issue, p. W219-W224.

192. Liu, T., Lin, Y., Wen, X., Jorissen, R. N., & Gilson, M. K. 2007, "BindingDB: a web-accessible database of experimentally determined protein-ligand binding affinities", *Nucleic Acids Res.*, vol. 35, no. Database issue, p. D198-D201.

193. Wang, R., Fang, X., Lu, Y., & Wang, S. 2004, "The PDBbind database: collection of binding affinities for protein-ligand complexes with known three-dimensional structures", *J.Med.Chem.*, vol. 47, no. 12, pp. 2977-2980.

194. Block, P., Sotriffer, C. A., Dramburg, I., & Klebe, G. 2006, "AffinDB: a freely accessible database of affinities for protein-ligand complexes from the PDB", *Nucleic Acids Res.*, vol. 34, no. Database issue, p. D522-D526.

195. Frank, R., Heikens, W., Heisterberg-Moutsis, G., & Blocker, H. 1983, "A new general approach for the simultaneous chemical synthesis of large numbers of oligonucleotides: segmental solid supports", *Nucleic Acids Res.*, vol. 11, no. 13, pp. 4365-4377.

196. Fodor, S. P., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T., & Solas, D. 1991, "Light-directed, spatially addressable parallel chemical synthesis", *Science*, vol. 251, no. 4995, pp. 767-773.

197. Frank, R. 2002, "The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications", *J.Immunol.Methods*, vol. 267, no. 1, pp. 13-26.

198. Reineke, U., Kramer, A., & Schneider-Mergener, J. 1999, "Antigen sequence- and library-based mapping of linear and discontinuous protein-protein-interaction sites by spot synthesis", *Curr.Top.Microbiol.Immunol.*, vol. 243, pp. 23-36.

199. Monnet, C., Laune, D., Laroche-Traineau, J., Biard-Piechaczyk, M., Briant, L., Bes, C., Pugniere, M., Mani, J. C., Pau, B., Cerutti, M., Devauchelle, G., Devaux, C., Granier, C., & Chardes, T. 1999, "Synthetic peptides derived from the variable regions of an anti-CD4 monoclonal antibody bind to CD4 and inhibit HIV-1 promoter activation in virus-infected cells", *J.Biol.Chem.*, vol. 274, no. 6, pp. 3789-3796.

200. Reineke, U., Sabat, R., Misselwitz, R., Welfle, H., Volk, H. D., & Schneider-Mergener, J. 1999, "A synthetic mimic of a discontinuous binding site on interleukin-10", *Nat.Biotechnol.*, vol. 17, no. 3, pp. 271-275.

201. Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S.,

Glockshuber, R., Riek, R., Billeter, M., Wuthrich, K., & Oesch, B. 1997, "Prion (PrPSc)-specific epitope defined by a monoclonal antibody", *Nature*, vol. 390, no. 6655, pp. 74-77.

202. Kramer, A., Stigler, R. D., Knaute, T., Hoffmann, B., & Schneider-Mergener, J. 1998, "Stepwise transformation of a cholera toxin and a p24 (HIV-1) epitope into D-peptide analogs", *Protein Eng*, vol. 11, no. 10, pp. 941-948.

203. Ball, L. J., Kuhne, R., Hoffmann, B., Hafner, A., Schmieder, P., Volkmer-Engert, R., Hof, M., Wahl, M., Schneider-Mergener, J., Walter, U., Oschkinat, H., & Jarchau, T. 2000, "Dual epitope recognition by the VASP EVH1 domain modulates polyproline ligand specificity and binding affinity", *EMBO J.*, vol. 19, no. 18, pp. 4903-4914.

204. Adler, S., Frank, R., Lanzavecchia, A., & Weiss, S. 1994, "T cell epitope analysis with peptides simultaneously synthesized on cellulose membranes: fine mapping of two DQ dependent epitopes", *FEBS Lett.*, vol. 352, no. 2, pp. 167-170.

205. Ay, B., Streitz, M., Boisguerin, P., Schlosser, A., Mahrenholz, C. C., Schuck, S. D., Kern, F., & Volkmer, R. 2007, "Sorting and pooling strategy: a novel tool to map a virus proteome for CD8 T-cell epitopes", *Biopolymers*, vol. 88, no. 1, pp. 64-75.

206. Tegge, W. J. & Frank, R. 1998, "Analysis of protein kinase substrate specificity by the use of peptide libraries on cellulose paper (SPOT-method)", *Methods Mol.Biol.*, vol. 87, pp. 99-106.

207. Ay, B., Landgraf, K., Streitz, M., Fuhrmann, S., Volkmer, R., & Boisguerin, P. 2008, "Using hydroxymethylphenoxy derivates with the SPOT technology to generate peptides with authentic C-termini", *Bioorg.Med.Chem.Lett.*, vol. 18, no. 14, pp. 4038-4043.

208. Mattioli, M. & Reichlin, M. 1974, "Heterogeneity of RNA protein antigens reactive with sera of patients with systemic lupus erythematosus. Description of a cytoplasmic nonribosomal antigen", *Arthritis Rheum.*, vol. 17, no. 4, pp. 421-429.

209. Alspaugh, M. A. & Tan, E. M. 1975, "Antibodies to cellular antigens in Sjogren's syndrome", *J.Clin.Invest*, vol. 55, no. 5, pp. 1067-1073.

210. Gottlieb, E. & Steitz, J. A. 1989, "The RNA binding protein La influences both the accuracy and the efficiency of RNA polymerase III transcription in vitro", *EMBO J.*, vol. 8, no. 3, pp. 841-850.

211. Fan, H., Goodier, J. L., Chamberlain, J. R., Engelke, D. R., & Maraia, R. J. 1998, "5' processing of tRNA precursors can Be modulated by the human La antigen phosphoprotein", *Mol.Cell Biol.*, vol. 18, no. 6, pp. 3201-3211.

212. Bai, C. & Tolias, P. P. 2000, "Genetic analysis of a La homolog in Drosophila melanogaster", *Nucleic Acids Res.*, vol. 28, no. 5, pp. 1078-1084.

213. Chambers, J. C., Kenan, D., Martin, B. J., & Keene, J. D. 1988, "Genomic structure and amino acid sequence domains of the human La autoantigen", *J.Biol.Chem.*, vol. 263, no. 34, pp. 18043-18051.

214. Lin-Marq, N. & Clarkson, S. G. 1995, "A yeast RNA binding protein that resembles the human autoantigen La", *J.Mol.Biol.*, vol. 245, no. 2, pp. 81-85.

215. Scherly, D., Stutz, F., Lin-Marq, N., & Clarkson, S. G. 1993, "La proteins from Xenopus laevis. cDNA cloning and developmental expression", *J.Mol.Biol.*, vol. 231, no. 2, pp. 196-204.

216. Van Horn, D. J., Yoo, C. J., Xue, D., Shi, H., & Wolin, S. L. 1997, "The La protein in Schizosaccharomyces pombe: a conserved yet dispensable phosphoprotein that functions in tRNA maturation", *RNA.*, vol. 3, no. 12, pp. 1434-1443.

217. Westermann, S. & Weber, K. 2000, "Cloning and recombinant expression of the La RNA-binding protein from Trypanosoma brucei", *Biochim.Biophys.Acta*, vol. 1492, no. 2-3, pp. 483-487.

218. Yoo, C. J. & Wolin, S. L. 1994, "La proteins from Drosophila melanogaster and Saccharomyces cerevisiae: a yeast homolog of the La autoantigen is dispensable for growth", *Mol.Cell Biol.*, vol. 14, no. 8, pp. 5412-5424.

219. Graus, F., Cordon-Cardo, C., Bonfa, E., & Elkon, K. B. 1985, "Immunohistochemical localization of La nuclear antigen in brain. Selective concentration of the La protein in neuronal nucleoli", *J.Neuroimmunol.*, vol. 9, no. 5, pp. 307-319.

220. Deng, J. S., Takasaki, Y., & Tan, E. M. 1981, "Nonhistone nuclear antigens reactive with autoantibodies. Immunofluorescence studies on distribution in synchronized cells", *J.Cell Biol.*, vol. 91, no. 3 Pt 1, pp. 654-660.

221. Sobel, S. G. & Wolin, S. L. 1999, "Two yeast La motif-containing proteins are RNA-binding proteins that associate with polyribosomes", *Mol.Biol.Cell*, vol. 10, no. 11, pp. 3849-3862.

222. Hendrick, J. P., Wolin, S. L., Rinke, J., Lerner, M. R., & Steitz, J. A. 1981, "Ro small cytoplasmic ribonucleoproteins are a subclass of La ribonucleoproteins: further characterization of the Ro and La small ribonucleoproteins from uninfected mammalian cells", *Mol.Cell Biol.*, vol. 1, no. 12, pp. 1138-1149.

223. Ayukawa, K., Taniguchi, S., Masumoto, J., Hashimoto, S., Sarvotham, H., Hara, A., Aoyama, T., & Sagara, J. 2000, "La autoantigen is cleaved in the COOH terminus and loses the nuclear localization signal during apoptosis", *J.Biol.Chem.*, vol. 275, no. 44, pp. 34465-34470.

224. Rutjes, S. A., Utz, P. J., van der, H. A., Broekhuis, C., van Venrooij, W. J., & Pruijn, G. J. 1999, "The La (SS-B) autoantigen, a key protein in RNA biogenesis, is dephosphorylated and cleaved early during apoptosis", *Cell Death.Differ.*, vol. 6, no. 10, pp. 976-986.

225. Waldsich, C., Grossberger, R., & Schroeder, R. 2002, "RNA chaperone StpA loosens interactions of the tertiary structure in the td group I intron in vivo", *Genes Dev.*, vol. 16, no. 17, pp. 2300-2312.

226. Herschlag, D. 1995, "RNA chaperones and the RNA folding problem", *J.Biol.Chem.*, vol. 270, no. 36, pp. 20871-20874.

227. Fan, H., Sakulich, A. L., Goodier, J. L., Zhang, X., Qin, J., & Maraia, R. J. 1997, "Phosphorylation of the human La antigen on serine 366 can regulate recycling of RNA polymerase III transcription complexes", *Cell*, vol. 88, no. 5, pp. 707-715.

228. Pannone, B. K., Xue, D., & Wolin, S. L. 1998, "A role for the yeast La protein in U6 snRNP assembly: evidence that the La protein is a molecular chaperone for RNA polymerase III transcripts", *EMBO J.*, vol. 17, no. 24, pp. 7442-7453.

229. Xue, D., Rubinson, D. A., Pannone, B. K., Yoo, C. J., & Wolin, S. L. 2000, "U snRNP assembly in yeast involves the La protein", *EMBO J.*, vol. 19, no. 7, pp. 1650-1660.

230. Yoo, C. J. & Wolin, S. L. 1997, "The yeast La protein is required for the 3' endonucleolytic cleavage that matures tRNA precursors", *Cell*, vol. 89, no. 3, pp. 393-402.

231. Meerovitch, K., Svitkin, Y. V., Lee, H. S., Lejbkowicz, F., Kenan, D. J., Chan, E. K., Agol, V. I., Keene, J. D., & Sonenberg, N. 1993, "La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate", *J.Virol.*, vol. 67, no. 7, pp. 3798-3807.

232. Wilusz, J., Kurilla, M. G., & Keene, J. D. 1983, "A host protein (La) binds to a unique species of minus-sense leader RNA during replication of vesicular stomatitis virus", *Proc.Natl.Acad.Sci.U.S.A*, vol. 80, no. 19, pp. 5827-5831.

233. Kurilla, M. G. & Keene, J. D. 1983, "The leader RNA of vesicular stomatitis virus is bound by a cellular protein reactive with anti-La lupus antibodies", *Cell*, vol. 34, no. 3, pp. 837-845.

234. Lerner, M. R., Boyle, J. A., Hardin, J. A., & Steitz, J. A. 1981, "Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus", *Science*, vol. 211, no. 4480, pp. 400-402.

235. Rosa, M. D., Gottlieb, E., Lerner, M. R., & Steitz, J. A. 1981, "Striking similarities are exhibited by two small Epstein-Barr virus-encoded ribonucleic acids and the adenovirus-associated ribonucleic acids VAI and VAII", *Mol.Cell Biol.*, vol. 1, no. 9, pp. 785-796.

236. Garcia-Montalvo, B. M., Medina, F., & del Angel, R. M. 2004, "La protein binds to NS5 and NS3 and to the 5' and 3' ends of Dengue 4 virus RNA", *Virus Res.*, vol. 102, no. 2, pp. 141-150.

237. Lerner, M. R., Andrews, N. C., Miller, G., & Steitz, J. A. 1981, "Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus", *Proc.Natl.Acad.Sci.U.S.A*, vol. 78, no. 2, pp. 805-809.

238. Fok, V., Friend, K., & Steitz, J. A. 2006, "Epstein-Barr virus noncoding RNAs are confined to the nucleus, whereas their partner, the human La protein, undergoes nucleocytoplasmic shuttling", *J.Cell Biol.*, vol. 173, no. 3, pp. 319-325.

239. Heise, T., Guidotti, L. G., Cavanaugh, V. J., & Chisari, F. V. 1999, "Hepatitis B virus RNA-binding proteins associated with cytokine-induced clearance of viral RNA from the liver of transgenic mice", *J.Virol.*, vol. 73, no. 1, pp. 474-481.

240. Pudi, R., Abhiman, S., Srinivasan, N., & Das, S. 2003, "Hepatitis C virus internal ribosome entry site-mediated translation is stimulated by specific interaction of independent regions of human La autoantigen", *J.Biol.Chem.*, vol. 278, no. 14, pp. 12231-12240.

241. Bachmann, M., Deister, H., Pautz, A., Laubinger, J., Schmitz, M., Falke, D., Podlech, J., & Grolz, D. 1998, "The human autoantigen La/SS-B accelerates herpes simplex virus type 1 replication in transfected mouse 3T3 cells", *Clin.Exp.Immunol.*, vol. 112, no. 3, pp. 482-489.

242. Chang, Y. N., Kenan, D. J., Keene, J. D., Gatignol, A., & Jeang, K. T. 1994, "Direct interactions between autoantigen La and human immunodeficiency virus leader RNA", *J.Virol.*, vol. 68, no. 11, pp. 7008-7020.

243. Gutierrez-Escolano, A. L., Vazquez-Ochoa, M., Escobar-Herrera, J., & Hernandez-Acosta, J. 2003, "La, PTB, and PAB proteins bind to the 3(') untranslated region of Norwalk virus genomic RNA", *Biochem.Biophys.Res.Commun.*, vol. 311, no. 3, pp. 759-766.

244. Svitkin, Y. V., Meerovitch, K., Lee, H. S., Dholakia, J. N., Kenan, D. J., Agol, V. I., & Sonenberg, N. 1994, "Internal translation initiation on poliovirus RNA: further characterization of La function in poliovirus translation in vitro", *J.Virol.*, vol. 68, no. 3, pp. 1544-1550.

245. Kim, Y. K., Back, S. H., Rho, J., Lee, S. H., & Jang, S. K. 2001, "La autoantigen enhances translation of BiP mRNA", *Nucleic Acids Res.*, vol. 29, no. 24, pp. 5009-5016.

246. McLaren, R. S., Caruccio, N., & Ross, J. 1997, "Human La protein: a stabilizer of histone mRNA", *Mol.Cell Biol.*, vol. 17, no. 6, pp. 3028-3036.

247. Steitz, J. A., Berg, C., Hendrick, J. P., La Branch, Metspalu, A., Rinke, J., & Yario, T. 1988, "A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells", *J.Cell Biol.*, vol. 106, no. 3, pp. 545-556.

248. Fan, H., Goodier, J. L., Chamberlain, J. R., Engelke, D. R., & Maraia, R. J. 1998, "5' processing of tRNA precursors can Be modulated by the human La antigen phosphoprotein", *Mol.Cell Biol.*, vol. 18, no. 6, pp. 3201-3211.

249. Yoo, C. J. & Wolin, S. L. 1997, "The yeast La protein is required for the 3' endonucleolytic cleavage that matures tRNA precursors", *Cell*, vol. 89, no. 3, pp. 393-402.

250. Intine, R. V., Sakulich, A. L., Koduru, S. B., Huang, Y., Pierstorff, E., Goodier, J. L., Phan, L., & Maraia, R. J. 2000, "Control of transfer RNA maturation by phosphorylation of the human La antigen on serine 366", *Mol.Cell*, vol. 6, no. 2, pp. 339-348.

251. Chakshusmathi, G., Kim, S. D., Rubinson, D. A., & Wolin, S. L. 2003, "A La protein requirement for efficient pre-tRNA folding", *EMBO J.*, vol. 22, no. 24, pp. 6562-6572.

252. Ford, L. P., Shay, J. W., & Wright, W. E. 2001, "The La antigen associates with the human telomerase ribonucleoprotein and influences telomere length in vivo", *RNA.*, vol. 7, no. 8, pp. 1068-1075.

253. Crosio, C., Boyl, P. P., Loreni, F., Pierandrei-Amaldi, P., & Amaldi, F. 2000, "La protein has a positive effect on the translation of TOP mRNAs in vivo", *Nucleic Acids Res.*, vol. 28, no. 15, pp. 2927-2934.

254. Kufel, J., Allmang, C., Chanfreau, G., Petfalski, E., Lafontaine, D. L., & Tollervey, D. 2000, "Precursors to the U3 small nucleolar RNA lack small nucleolar RNP proteins but are stabilized by La binding", *Mol.Cell Biol.*, vol. 20, no. 15, pp. 5415-5424.

255. Xue, D., Rubinson, D. A., Pannone, B. K., Yoo, C. J., & Wolin, S. L. 2000, "U snRNP assembly in yeast involves the La protein", *EMBO J.*, vol. 19, no. 7, pp. 1650-1660.

256. Rinke, J. & Steitz, J. A. 1985, "Association of the lupus antigen La with a subset of U6 snRNA molecules", *Nucleic Acids Res.*, vol. 13, no. 7, pp. 2617-2629.

257. Kickhoefer, V. A., Poderycki, M. J., Chan, E. K., & Rome, L. H. 2002, "The La RNA-binding protein interacts with the vault RNA and is a vault-associated protein", *J.Biol.Chem.*, vol. 277, no. 43, pp. 41282-41286.

258. Rutjes, S. A., van der, H. A., Utz, P. J., van Venrooij, W. J., & Pruijn, G. J. 1999, "Rapid nucleolytic degradation of the small cytoplasmic Y RNAs during apoptosis", *J.Biol.Chem.*, vol. 274, no. 35, pp. 24799-24807.

259. Custer, B., Sullivan, S. D., Hazlet, T. K., Iloeje, U., Veenstra, D. L., & Kowdley, K. V. 2004, "Global epidemiology of hepatitis B virus", *J.Clin.Gastroenterol.*, vol. 38, no. 10 Suppl, p. S158-S168.

260. Guidotti, L. G. & Chisari, F. V. 2000, "Cytokine-mediated control of viral infections", *Virology*, vol. 273, no. 2, pp. 221-227.

261. Heise, T., Guidotti, L. G., & Chisari, F. V. 1999, "La autoantigen specifically recognizes a predicted stem-loop in hepatitis B virus RNA", *J.Virol.*, vol. 73, no. 7, pp. 5767-5776.

262. Szmuness, W., Stevens, C. E., Zang, E. A., Harley, E. J., & Kellner, A. 1981, "A controlled clinical trial of the efficacy of the hepatitis B vaccine (Heptavax B): a final report", *Hepatology*, vol. 1, no. 5, pp. 377-385.

263. Poland, G. A. & Jacobson, R. M. 2004, "Clinical practice: prevention of hepatitis B with the hepatitis B vaccine", *N.Engl.J.Med.*, vol. 351, no. 27, pp. 2832-2838.

264. Lu, M. & Lorentz, T. 2003, "De novo infection in a renal transplant recipient caused by novel mutants of hepatitis B virus despite the presence of protective anti-hepatitis B surface antibody", *J.Infect.Dis.*, vol. 187, no. 8, pp. 1323-1326.

265. Rivkina, A. & Rybalov, S. 2002, "Chronic hepatitis B: current and future treatment options",
Pharmacotherapy, vol. 22, no. 6, pp. 721-737.

266. Radhakrishnan, S. K., Layden, T. J., & Gartel, A. L. 2004, "RNA interference as a new strategy against viral hepatitis", *Virology*, vol. 323, no. 2, pp. 173-181.

267. Zhao, Z. F., Yang, H., Han, D. W., Zhao, L. F., Zhang, G. Y., Zhang, Y., & Liu, M. S. 2006, "Inhibition of hepatitis B virus expression and replication by RNA interference in HepG2.2.15", *World J.Gastroenterol.*, vol. 12, no. 37, pp. 6046-6049.

268. Zhang, X. N., Xiong, W., Wang, J. D., Hu, Y. W., Xiang, L., & Yuan, Z. H. 2004, "siRNA-mediated inhibition of HBV replication and expression", *World J.Gastroenterol.*, vol. 10, no. 20, pp. 2967-2971.

269. Ying, R. S., Zhu, C., Fan, X. G., Li, N., Tian, X. F., Liu, H. B., & Zhang, B. X. 2007, "Hepatitis B virus is inhibited by RNA interference in cell culture and in mice", *Antiviral Res.*, vol. 73, no. 1, pp. 24-30.

270. McCaffrey, A. P., Nakai, H., Pandey, K., Huang, Z., Salazar, F. H., Xu, H., Wieland, S. F., Marion, P. L., & Kay, M. A. 2003, "Inhibition of hepatitis B virus in mice by RNA interference", *Nat.Biotechnol.*, vol. 21, no. 6, pp. 639-644.

271. Ni, Q., Chen, Z., Yao, H. P., Yang, Z. G., Liu, K. Z., & Wu, L. L. 2004, "Inhibition of human La protein by RNA interference downregulates hepatitis B virus mRNA in 2.2.15 cells", *World J.Gastroenterol.*, vol. 10, no. 14, pp. 2050-2054.

272. Horke, S., Reumann, K., Rang, A., & Heise, T. 2002, "Molecular characterization of the human La protein.hepatitis B virus RNA.B interaction in vitro", *J.Biol.Chem.*, vol. 277, no. 38, pp. 34949-34958.

273. Horke, S., Reumann, K., Schweizer, M., Will, H., & Heise, T. 2004, "Nuclear trafficking of La protein depends on a newly identified nucleolar localization signal and the ability to bind RNA", *J.Biol.Chem.*, vol. 279, no. 25, pp. 26563-26570.

274. Horke, S., Reumann, K., Schulze, C., Grosse, F., & Heise, T. 2004, "The La motif and the RNA recognition motifs of human La autoantigen contribute individually to RNA recognition and subcellular localization", *J.Biol.Chem.*, vol. 279, no. 48, pp. 50302-50309.

275. Honda, A., Adams, S. R., Sawyer, C. L., Lev-Ram, V., Tsien, R. Y., & Dostmann, W. R. 2001, "Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator", *Proc.Natl.Acad.Sci.U.S.A*, vol. 98, no. 5, pp. 2437-2442.

276. Glasel, J. A. 1995, "Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios", *Biotechniques*, vol. 18, no. 1, pp. 62-63.

277. Laemmli, U. K. 1970, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature*, vol. 227, no. 5259, pp. 680-685.

278. Heukeshoven, J. & Dernick, R. 1988, "Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels", *Electrophoresis*, vol. 9, no. 1, pp. 28-32.

279. Dostmann, W. R., Tegge, W., Frank, R., Nickl, C. K., Taylor, M. S., & Brayden, J. E. 2002, "Exploring the mechanisms of vascular smooth muscle tone with highly specific, membrane-permeable inhibitors of cyclic GMP-dependent protein kinase Ialpha", *Pharmacol.Ther.*, vol. 93, no. 2-3, pp. 203-215.

280. Nakagawa, H., Miki, H., Ito, M., Ohashi, K., Takenawa, T., & Miyamoto, S. 2001, "N-WASP, WAVE and Mena play different roles in the organization of actin cytoskeleton in lamellipodia", *J.Cell Sci.*, vol. 114, no. Pt 8, pp. 1555-1565.

281. Dostmann, W. R., Tegge, W., Frank, R., Nickl, C. K., Taylor, M. S., & Brayden, J. E. 2002, "Exploring the mechanisms of vascular smooth muscle tone with highly specific, membrane-permeable inhibitors of cyclic GMP-dependent protein kinase Ialpha", *Pharmacol.Ther.*, vol. 93, no. 2-3, pp. 203-215.

282. Heger, P., Lohmaier, J., Schneider, G., Schweimer, K., & Stauber, R. H. 2001, "Qualitative highly divergent nuclear export signals can regulate export by the competition for transport cofactors in vivo", *Traffic.*, vol. 2, no. 8, pp. 544-555.

283. Uyar, D., Takigawa, N., Mekhail, T., Grabowski, D., Markman, M., Lee, F., Canetta, R., Peck, R., Bukowski, R., & Ganapathi, R. 2003, "Apoptotic pathways of epothilone BMS 310705", *Gynecol.Oncol.*, vol. 91, no. 1, pp. 173-178.

284. Chan, E. K., Francoeur, A. M., & Tan, E. M. 1986, "Epitopes, structural domains, and asymmetry of amino acid residues in SS-B/La nuclear protein", *J.Immunol.*, vol. 136, no. 10, pp. 3744-3749.

285. Cao, T., Wang, A., Liang, X., Tang, H., Auner, G. W., Salley, S. O., & Simon Ng, K. Y. 2007, "Investigation of spacer length effect on immobilized Escherichia coli pili-antibody molecular recognition by AFM", *Biotechnol.Bioeng.*

286. Everaerts, F., Gillissen, M., Torrianni, M., Zilla, P., Human, P., Hendriks, M., & Feijen, J. 2006, "Reduction of calcification of carbodiimide-processed heart valve tissue by prior blocking of amine groups with monoaldehydes", *J.Heart Valve Dis.*, vol. 15, no. 2, pp. 269-277.

287. Everaerts, F., Torrianni, M., van Luyn, M., van Wachem, P., Feijen, J., & Hendriks, M. 2004, "Reduced calcification of bioprostheses, cross-linked via an improved carbodiimide based method", *Biomaterials*, vol. 25, no. 24, pp. 5523-5530.

288. Small, J. V., Stradal, T., Vignal, E., & Rottner, K. 2002, "The lamellipodium: where motility begins", *Trends Cell Biol.*, vol. 12, no. 3, pp. 112-120.

289. Lakard, S., Herlem, G., Propper, A., Kastner, A., Michel, G., Valles-Villarreal, N., Gharbi, T., & Fahys, B. 2004, "Adhesion and proliferation of cells on new polymers modified biomaterials", *Bioelectrochemistry.*, vol. 62, no. 1, pp. 19-27.

290. Pelham, R. J., Jr. & Wang, Y. 1997, "Cell locomotion and focal adhesions are regulated by substrate flexibility", *Proc.Natl.Acad.Sci.U.S.A*, vol. 94, no. 25, pp. 13661-13665.

291. Small, J. V., Rottner, K., & Kaverina, I. 1999, "Functional design in the actin cytoskeleton", *Curr.Opin.Cell Biol.*, vol. 11, no. 1, pp. 54-60.

292. Defilippi, P., Olivo, C., Tarone, G., Mancini, P., Torrisi, M. R., & Eva, A. 1997, "Actin cytoskeleton polymerization in Dbl-transformed NIH3T3 fibroblasts is dependent on cell adhesion to specific extracellular matrix proteins", *Oncogene*, vol. 14, no. 16, pp. 1933-1943.

293. Elnakady, Y. A., Sasse, F., Lunsdorf, H., & Reichenbach, H. 2004, "Disorazol A1, a highly effective antimitotic agent acting on tubulin polymerization and inducing apoptosis in mammalian cells", *Biochem.Pharmacol.*, vol. 67, no. 5, pp. 927-935.

294. Jackson, J. R., Patrick, D. R., Dar, M. M., & Huang, P. S. 2007, "Targeted anti-mitotic therapies: can we improve on tubulin agents?", *Nat.Rev.Cancer*, vol. 7, no. 2, pp. 107-117.

295. Dostmann, W. R., Taylor, M. S., Nickl, C. K., Brayden, J. E., Frank, R., & Tegge, W. J. 2000, "Highly specific, membrane-permeant peptide blockers of cGMP-dependent protein kinase Ialpha inhibit NO-induced cerebral dilation", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 26, pp. 14772-14777.

296. Morris, M. C., Depollier, J., Mery, J., Heitz, F., & Divita, G. 2001, "A peptide carrier for the delivery of biologically active proteins into mammalian cells", *Nat Biotechnol.*, vol. 19, no. 12, pp. 1173-1176.

297. Avrameas, A., Ternynck, T., Gasmi, L., & Buttin, G. 1999, "Efficient gene delivery by a peptide derived from a monoclonal anti-DNA antibody", *Bioconjug.Chem.*, vol. 10, no. 1, pp. 87-93.

298. Avrameas, A., Ternynck, T., Nato, F., Buttin, G., & Avrameas, S. 1998, "Polyreactive anti-DNA monoclonal antibodies and a derived peptide as vectors for the intracytoplasmic and intranuclear translocation of macromolecules", *Proc.Natl.Acad.Sci.U.S.A*, vol. 95, no. 10, pp. 5601-5606.

299. Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G., & Prochiantz, A. 1996, "Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent", *J.Biol.Chem.*, vol. 271, no. 30, pp. 18188-18193.

300. Elliott, G. & O'Hare, P. 1997, "Intercellular trafficking and protein delivery by a herpesvirus structural protein", *Cell*, vol. 88, no. 2, pp. 223-233.

301. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., & Barsoum, J. 1994, "Tat-mediated delivery of heterologous proteins into cells", *Proc.Natl.Acad.Sci.U.S.A*, vol. 91, no. 2, pp. 664-668.

302. Vives, E., Brodin, P., & Lebleu, B. 1997, "A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus", *J.Biol.Chem.*, vol. 272, no. 25, pp. 16010-16017.

303. Joliot, A. & Prochiantz, A. 2004, "Transduction peptides: from technology to physiology", *Nat.Cell Biol.*, vol. 6, no. 3, pp. 189-196.

304. Forster, T. 1948. Intramolecular energy migration and fluorescence.Ann Phys.(Leipzig) 2, 55-75.

305. Pollok, B. A. & Heim, R. 1999, "Using GFP in FRET-based applications", *Trends Cell Biol.*, vol. 9, no. 2, pp. 57-60.

306. Tsien, R. Y. 1998, "The green fluorescent protein", *Annu.Rev.Biochem.*, vol. 67, pp. 509-544.

307. Heim, R. & Tsien, R. Y. 1996, "Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer", *Curr.Biol.*, vol. 6, no. 2, pp. 178-182.

308. Heim, R. 1999, "Green fluorescent protein forms for energy transfer", *Methods Enzymol.*, vol. 302, pp. 408-423.

309. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., & Tsien, R. Y. 1997, "Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin", *Nature*, vol. 388, no. 6645, pp. 882-887.

310. Heim, R., Prasher, D. C., & Tsien, R. Y. 1994, "Wavelength mutations and posttranslational autoxidation of green fluorescent protein", *Proc.Natl.Acad.Sci.U.S.A*, vol. 91, no. 26, pp. 12501-12504.

311. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., & Tsien, R. Y. 1995, "Understanding, improving and using green fluorescent proteins", *Trends Biochem.Sci.*, vol. 20, no. 11, pp. 448-455.

312. Truong, K. & Ikura, M. 2001, "The use of FRET imaging microscopy to detect protein-protein interactions and protein conformational changes in vivo", *Curr.Opin.Struct.Biol.*, vol. 11, no. 5, pp. 573-578.

313. Wouters, F. S., Verveer, P. J., & Bastiaens, P. I. 2001, "Imaging biochemistry inside cells", *Trends Cell Biol.*, vol. 11, no. 5, pp. 203-211.

314. Bunt, G. & Wouters, F. S. 2004, "Visualization of molecular activities inside living cells with fluorescent labels", *Int.Rev.Cytol.*, vol. 237, pp. 205-277.

315. Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., & Tsien, R. Y. 2004, "Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein", *Nat.Biotechnol.*, vol. 22, no. 12, pp. 1567-1572.

316. Tsien, R. Y. 2006, "Breeding and building molecules to spy on cells and tumors", *Keio J.Med.*, vol. 55, no. 4, pp. 127-140.

317. Lissandron, V., Terrin, A., Collini, M., D'alfonso, L., Chirico, G., Pantano, S., & Zaccolo, M. 2005, "Improvement of a FRET-based indicator for cAMP by linker design and stabilization of donor-acceptor interaction", *J.Mol.Biol.*, vol. 354, no. 3, pp. 546-555.

318. Lissandron, V., Rossetto, M. G., Erbguth, K., Fiala, A., Daga, A., & Zaccolo, M. 2007, "Transgenic fruit-flies expressing a FRET-based sensor for in vivo imaging of cAMP dynamics", *Cell Signal.*, vol. 19, no. 11, pp. 2296-2303.

319. Persechini, A. & Cronk, B. 1999, "The relationship between the free concentrations of Ca2+ and Ca2+-calmodulin in intact cells", *J.Biol.Chem.*, vol. 274, no. 11, pp. 6827-6830.

320. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., & Tsien, R. Y. 1997, "Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin", *Nature*, vol. 388, no. 6645, pp. 882-887.

321. Kurokawa, K., Mochizuki, N., Ohba, Y., Mizuno, H., Miyawaki, A., & Matsuda, M. 2001, "A pair of fluorescent resonance energy transfer-based probes for tyrosine phosphorylation of the CrkII adaptor protein in vivo", *J.Biol.Chem.*, vol. 276, no. 33, pp. 31305-31310.

322. Kurokawa, K., Mochizuki, N., Ohba, Y., Mizuno, H., Miyawaki, A., & Matsuda, M. 2001, "A pair of fluorescent resonance energy transfer-based probes for tyrosine phosphorylation of the CrkII adaptor protein in vivo", *J.Biol.Chem.*, vol. 276, no. 33, pp. 31305-31310.

323. Ting, A. Y., Kain, K. H., Klemke, R. L., & Tsien, R. Y. 2001, "Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells", *Proc.Natl.Acad.Sci.U.S.A*, vol. 98, no. 26, pp. 15003-15008.

324. Kurokawa, K., Itoh, R. E., Yoshizaki, H., Nakamura, Y. O., & Matsuda, M. 2004, "Coactivation of Rac1 and Cdc42 at lamellipodia and membrane ruffles induced by epidermal growth factor", *Mol.Biol.Cell*, vol. 15, no. 3, pp. 1003-1010.

325. Jiao, X., Zhang, N., Xu, X., Oppenheim, J. J., & Jin, T. 2005, "Ligand-induced partitioning of human CXCR1 chemokine receptors with lipid raft microenvironments facilitates G-protein-dependent signaling", *Mol.Cell Biol.*, vol. 25, no. 13, pp. 5752-5762.

326. Damelin, M. & Silver, P. A. 2000, "Mapping interactions between nuclear transport factors in living cells reveals pathways through the nuclear pore complex", *Mol.Cell*, vol. 5, no. 1, pp. 133-140.

327. Hayes, M. J., Shao, D., Bailly, M., & Moss, S. E. 2006, "Regulation of actin dynamics by annexin 2", *EMBO J.*, vol. 25, no. 9, pp. 1816-1826.

328. Kuningas, K., Ukonaho, T., Pakkila, H., Rantanen, T., Rosenberg, J., Lovgren, T., & Soukka, T. 2006, "Upconversion fluorescence resonance energy transfer in a homogeneous immunoassay for estradiol", *Anal.Chem.*, vol. 78, no. 13, pp. 4690-4696.

329. Li, E., You, M., & Hristova, K. 2005, "Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and forster resonance energy transfer suggest weak interactions between fibroblast growth factor receptor 3 (FGFR3) transmembrane domains in the absence of extracellular domains and ligands", *Biochemistry*, vol. 44, no. 1, pp. 352-360.

330. Bu, P., Zhuang, J., Feng, J., Yang, D., Shen, X., & Yan, X. 2007, "Visualization of CD146 dimerization and its regulation in living cells", *Biochim.Biophys.Acta*, vol. 1773, no. 4, pp. 513-520.

331. Yates, R. M., Hermetter, A., & Russell, D. G. 2005, "The kinetics of phagosome maturation as a function of phagosome/lysosome fusion and acquisition of hydrolytic activity", *Traffic.*, vol. 6, no. 5, pp. 413-420.

332. Gaibelet, G., Planchenault, T., Mazeres, S., Dumas, F., Arenzana-Seisdedos, F., Lopez, A., Lagane, B., & Bachelerie, F. 2006, "CD4 and CCR5 constitutively interact at the plasma membrane of living cells: a confocal fluorescence resonance energy transfer-based approach", *J.Biol.Chem.*, vol. 281, no. 49, pp. 37921-37929.

333. Hwang, Y. C., Chen, W., & Yates, M. V. 2006, "Use of fluorescence resonance energy transfer for rapid detection of enteroviral infection in vivo", *Appl.Environ.Microbiol.*, vol. 72, no. 5, pp. 3710-3715.

334. Murata, S., Herman, P., Iwashina, M., Mochizuki, K., Nakazawa, T., Kondo, T., Nakamura, N., Lakowicz, J. R., & Katoh, R. 2005, "Application of microscopic Forster resonance energy transfer to cytological diagnosis of the thyroid tumors", *J.Biomed.Opt.*, vol. 10, no. 3, p. 034008.

335. Siegel, R. M., Frederiksen, J. K., Zacharias, D. A., Chan, F. K., Johnson, M., Lynch, D., Tsien, R. Y., & Lenardo, M. J. 2000, "Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations", *Science*, vol. 288, no. 5475, pp. 2354-2357.

336. Wu, X., Simone, J., Hewgill, D., Siegel, R., Lipsky, P. E., & He, L. 2006, "Measurement of two caspase activities simultaneously in living cells by a novel dual FRET fluorescent indicator probe", *Cytometry A*, vol. 69, no. 6, pp. 477-486.

337. Chun, W. & Johnson, G. V. 2007, "Activation of glycogen synthase kinase 3beta promotes the intermolecular association of tau. The use of fluorescence resonance energy transfer microscopy", *J.Biol.Chem.*, vol. 282, no. 32, pp. 23410-23417.

338. Karpova, T. S., Baumann, C. T., He, L., Wu, X., Grammer, A., Lipsky, P., Hager, G. L., & McNally, J. G. 2003, "Fluorescence resonance energy transfer from cyan to yellow fluorescent protein detected by acceptor photobleaching using confocal microscopy and a single laser", *J.Microsc.*, vol. 209, no. Pt 1, pp. 56-70.

339. van Munster, E. B., Kremers, G. J., Adjobo-Hermans, M. J., & Gadella, T. W., Jr. 2005, "Fluorescence resonance energy transfer (FRET) measurement by gradual acceptor photobleaching", *J.Microsc.*, vol. 218, no. Pt 3, pp. 253-262.

340. Ayukawa, K., Taniguchi, S., Masumoto, J., Hashimoto, S., Sarvotham, H., Hara, A., Aoyama, T., & Sagara, J. 2000, "La autoantigen is cleaved in the COOH terminus and loses the nuclear localization signal during apoptosis", *J.Biol.Chem.*, vol. 275, no. 44, pp. 34465-34470.

341. Uyar, D., Takigawa, N., Mekhail, T., Grabowski, D., Markman, M., Lee, F., Canetta, R., Peck, R., Bukowski, R., & Ganapathi, R. 2003, "Apoptotic pathways of epothilone BMS 310705", *Gynecol.Oncol.*, vol. 91, no. 1, pp. 173-178.

342. Altmann, K. H., Wartmann, M., & O'Reilly, T. 2000, "Epothilones and related structures--a new class of microtubule inhibitors with potent in vivo antitumor activity", *Biochim.Biophys.Acta*, vol. 1470, no. 3, p. M79-M91.

343. Ayukawa, K., Taniguchi, S., Masumoto, J., Hashimoto, S., Sarvotham, H., Hara, A., Aoyama, T., & Sagara, J. 2000, "La autoantigen is cleaved in the COOH terminus and loses the nuclear localization signal during apoptosis", *J.Biol.Chem.*, vol. 275, no. 44, pp. 34465-34470.

6 Appendices

6.1 Behaviour of cells to patch chemistry

Figure 6-1 to figure 6-10 compare cells grown on PP foils with different chemical functions (see Appendix 6.3 for more details).

The left panels indicate cells grown on patches and the right panels are cells on patches stained with Trypan blue.

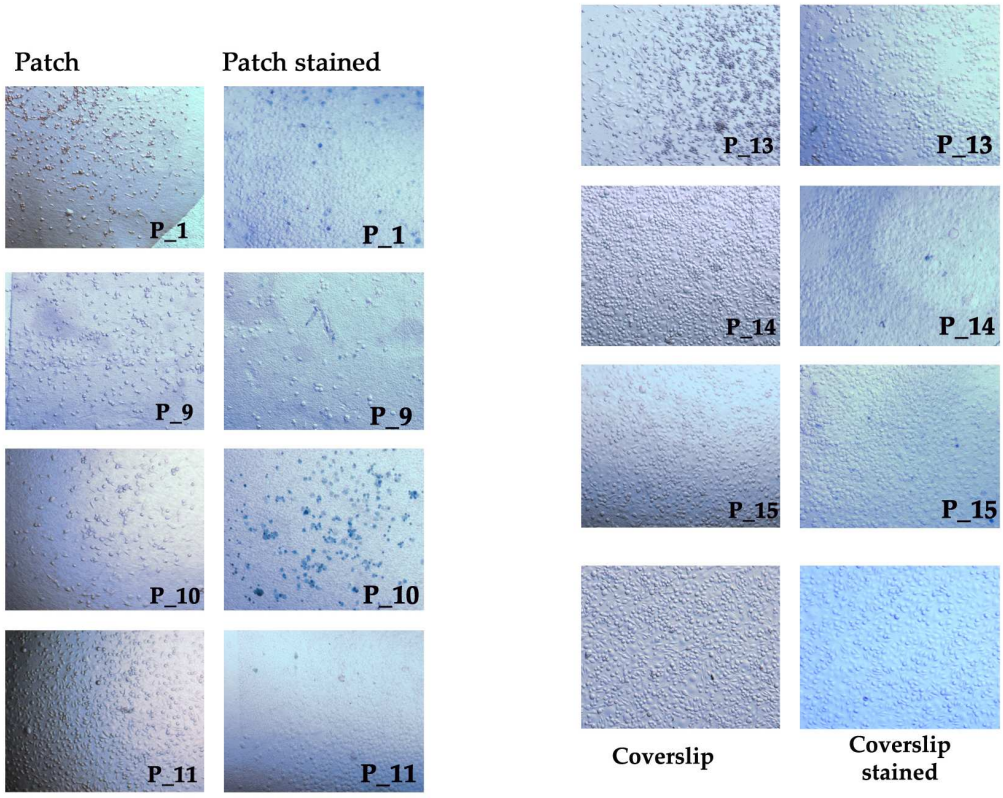

Figure 6-1. Behaviour of L929 cells with respect to patch chemistry

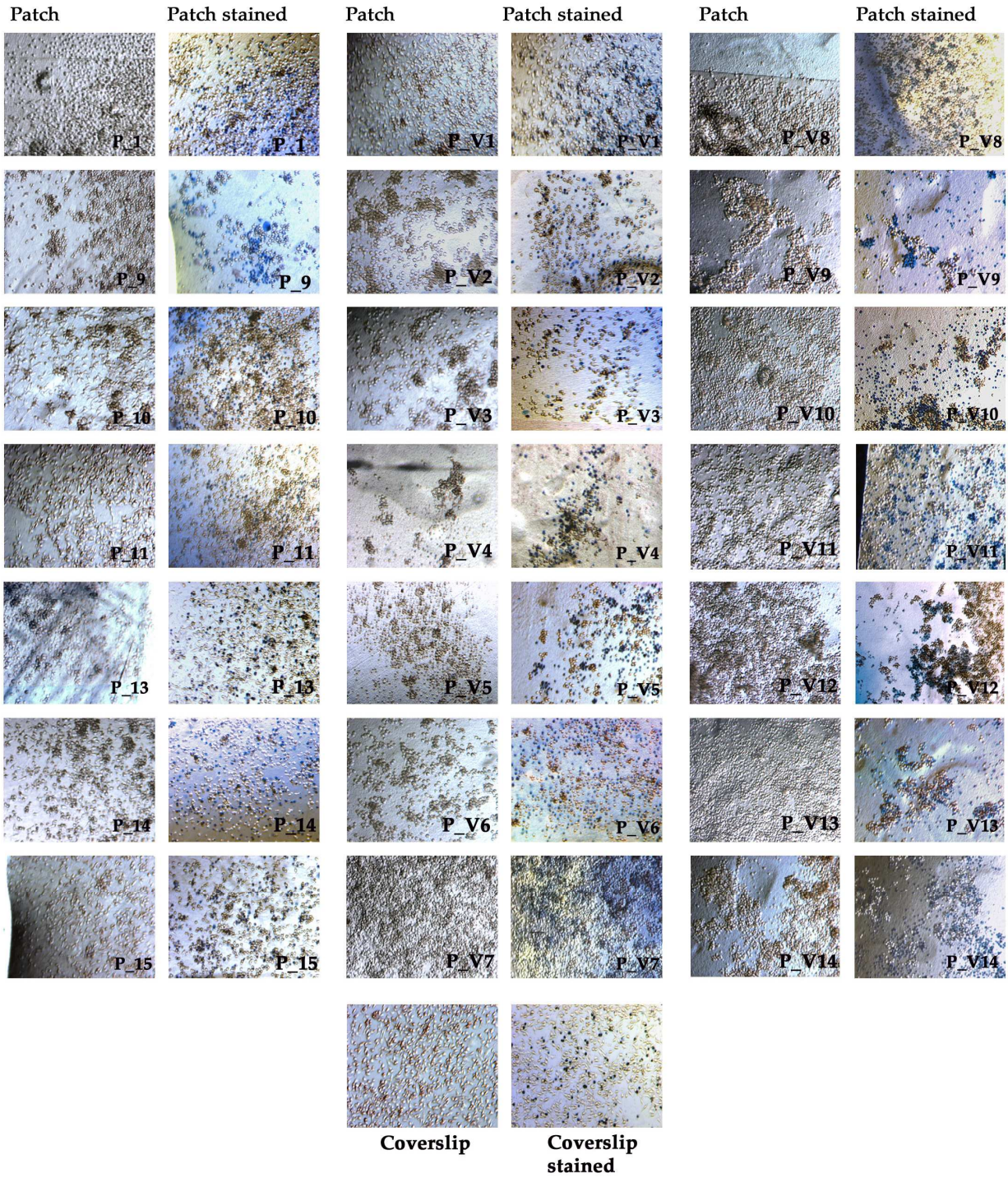

Figure 6-2. Behaviour of HeLa cells with respect to patch chemistry

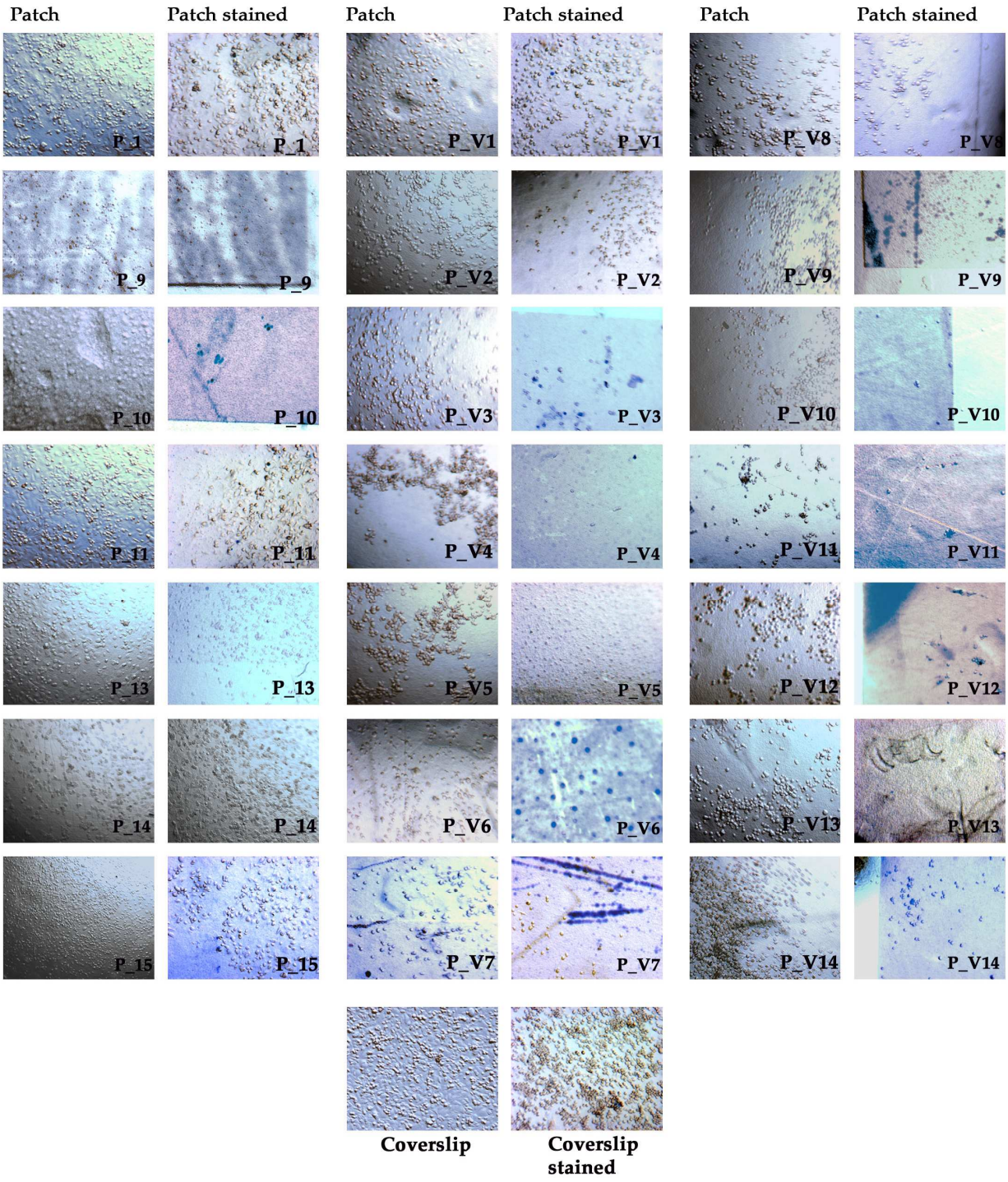

Figure 6-3. Behaviour of SKOV3 cells with respect to patch chemistry

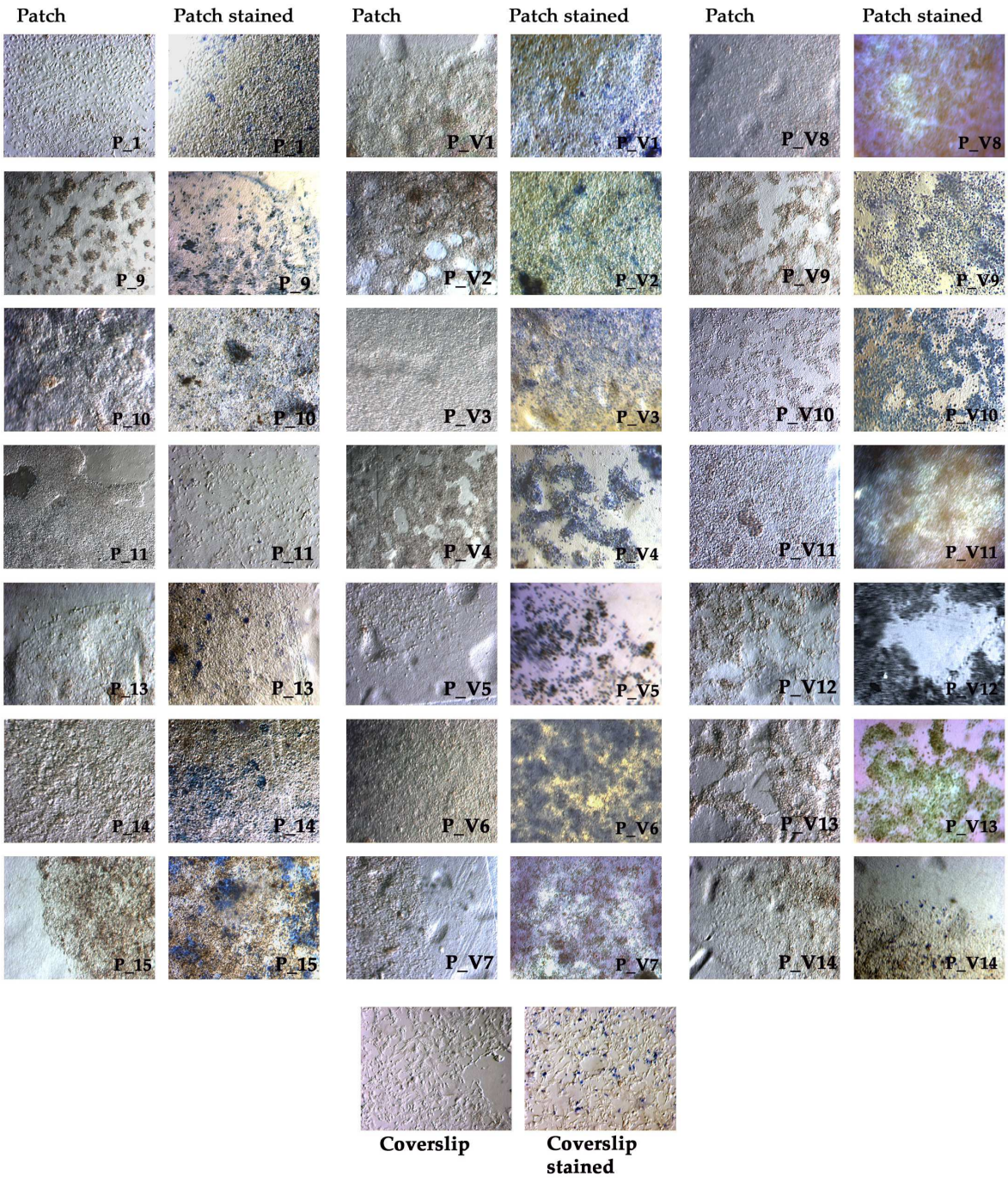

Figure 6-4. Behaviour of Huh7 cells with respect to patch chemistry

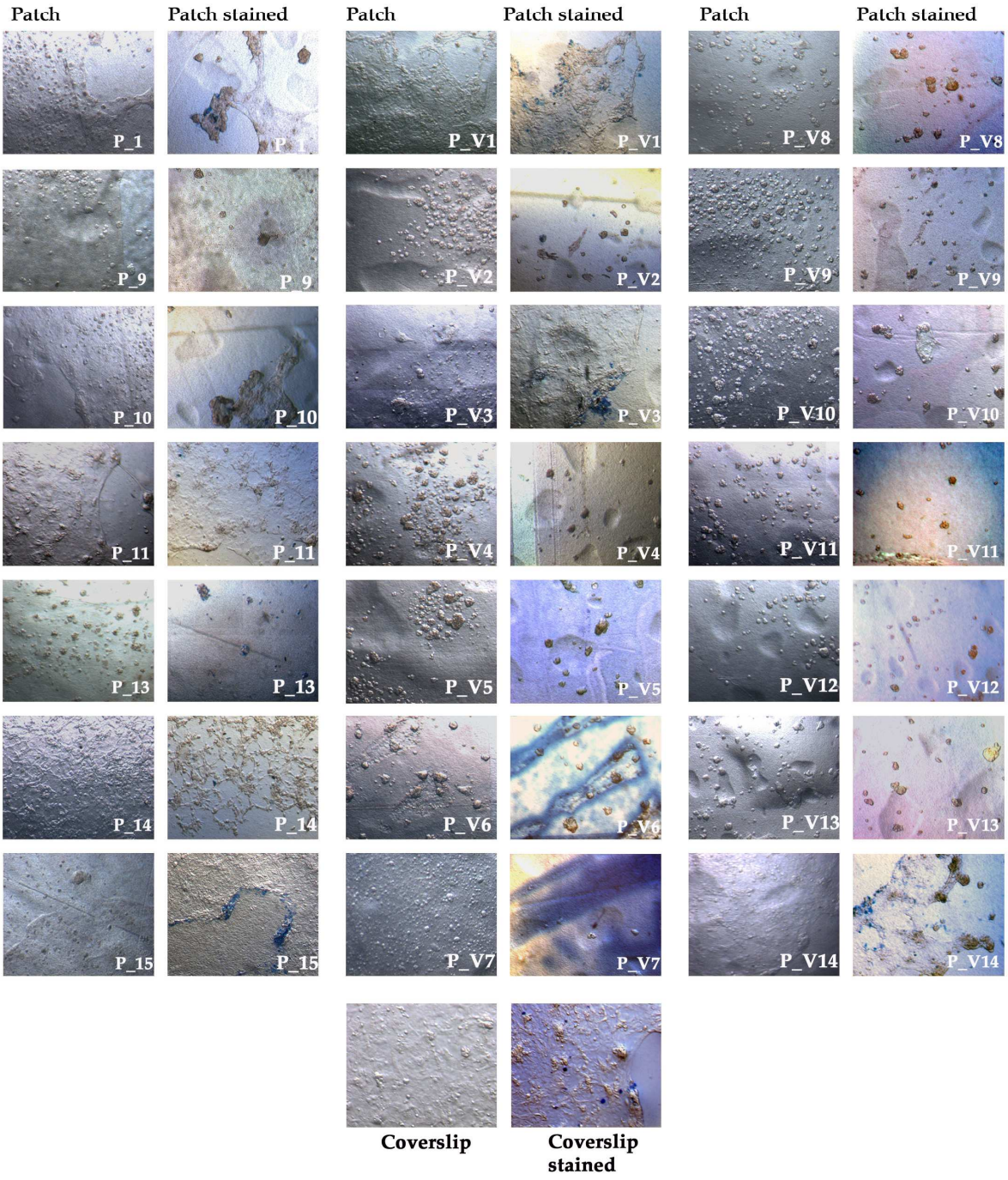

Figure 6-5. Behaviour of A431 cells with respect to patch chemistry

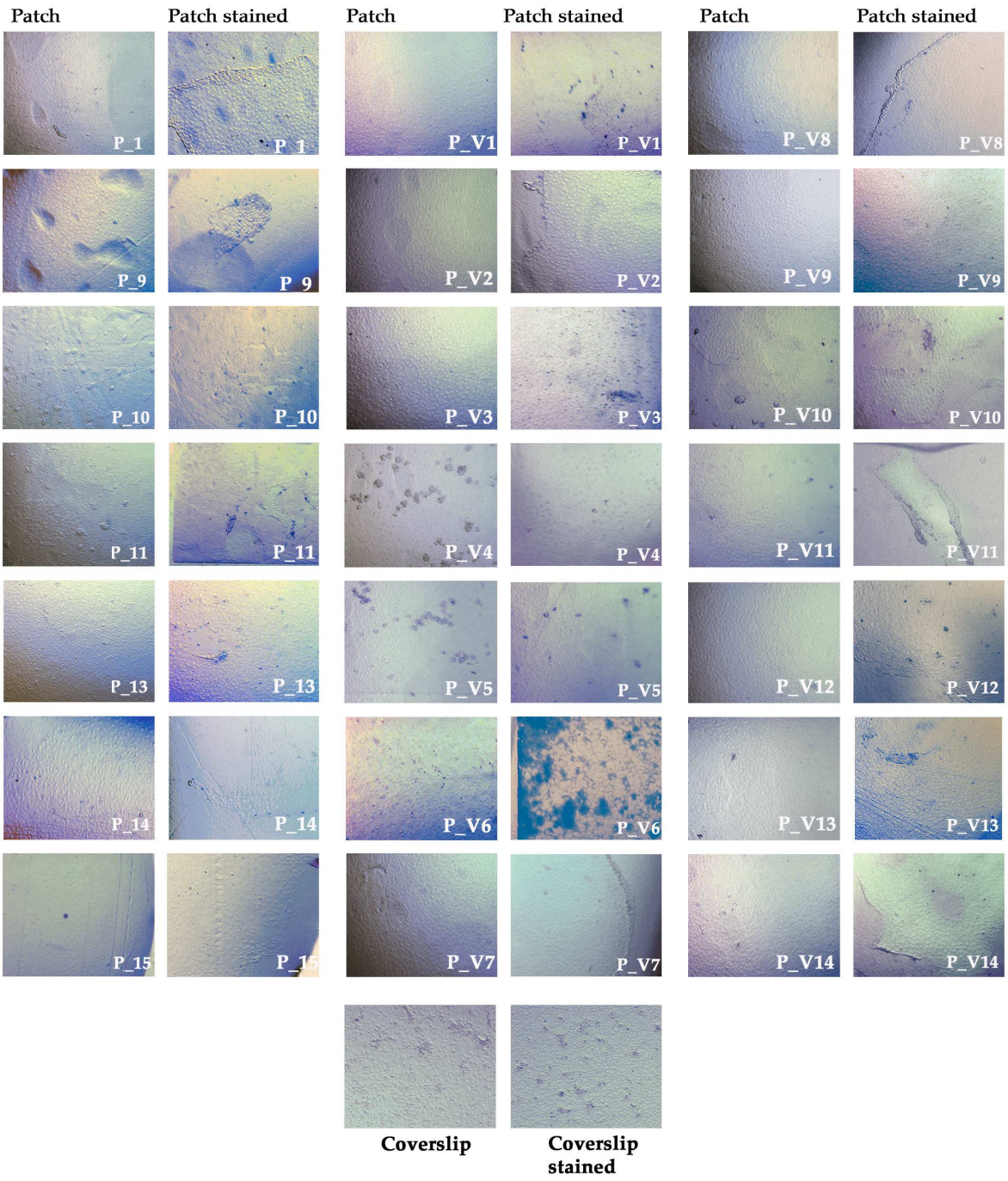

Figure 6-6. Behaviour of A498 cells with respect to patch chemistry

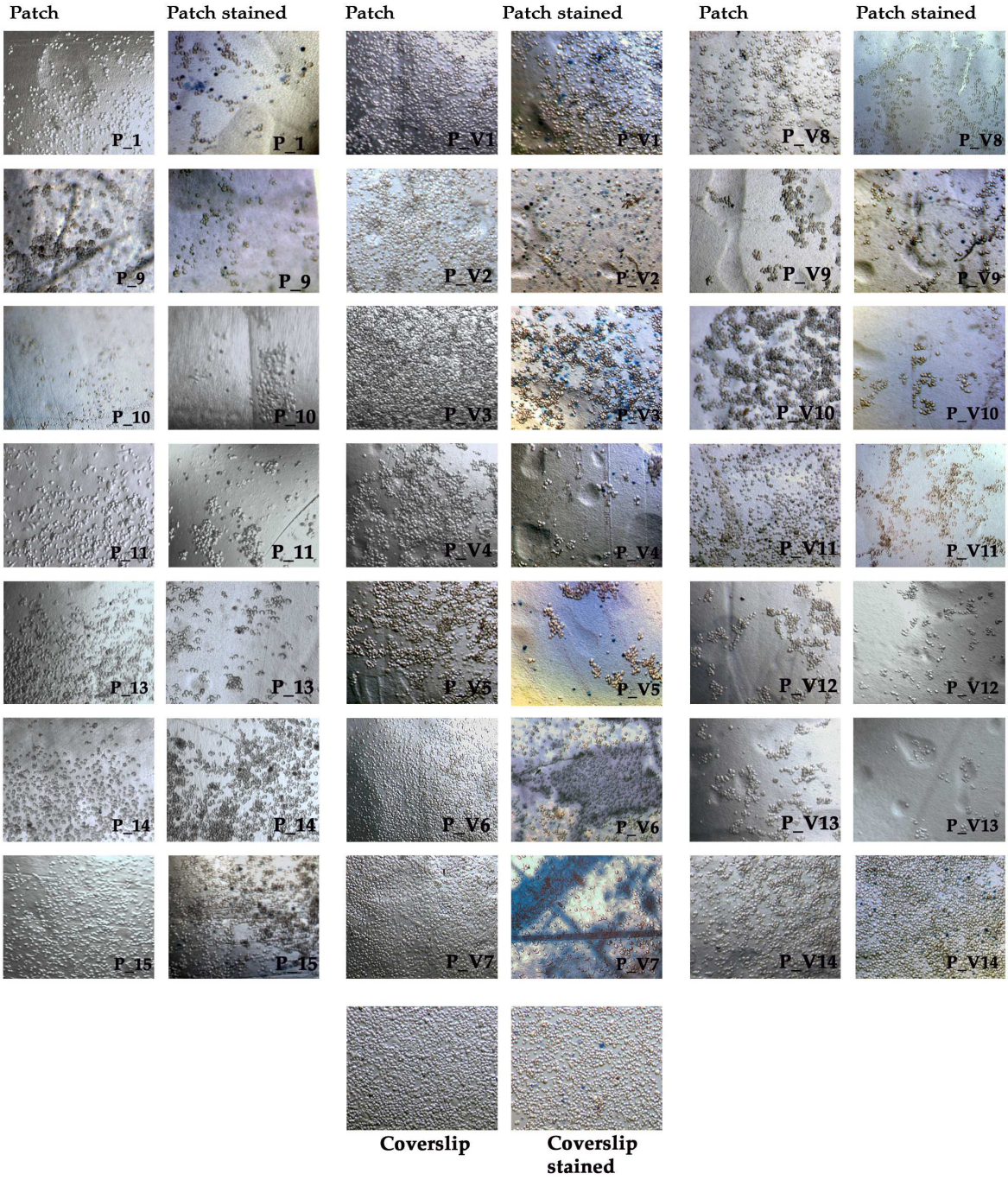

Figure 6-7. Behaviour of PC3 cells with respect to patch chemistry

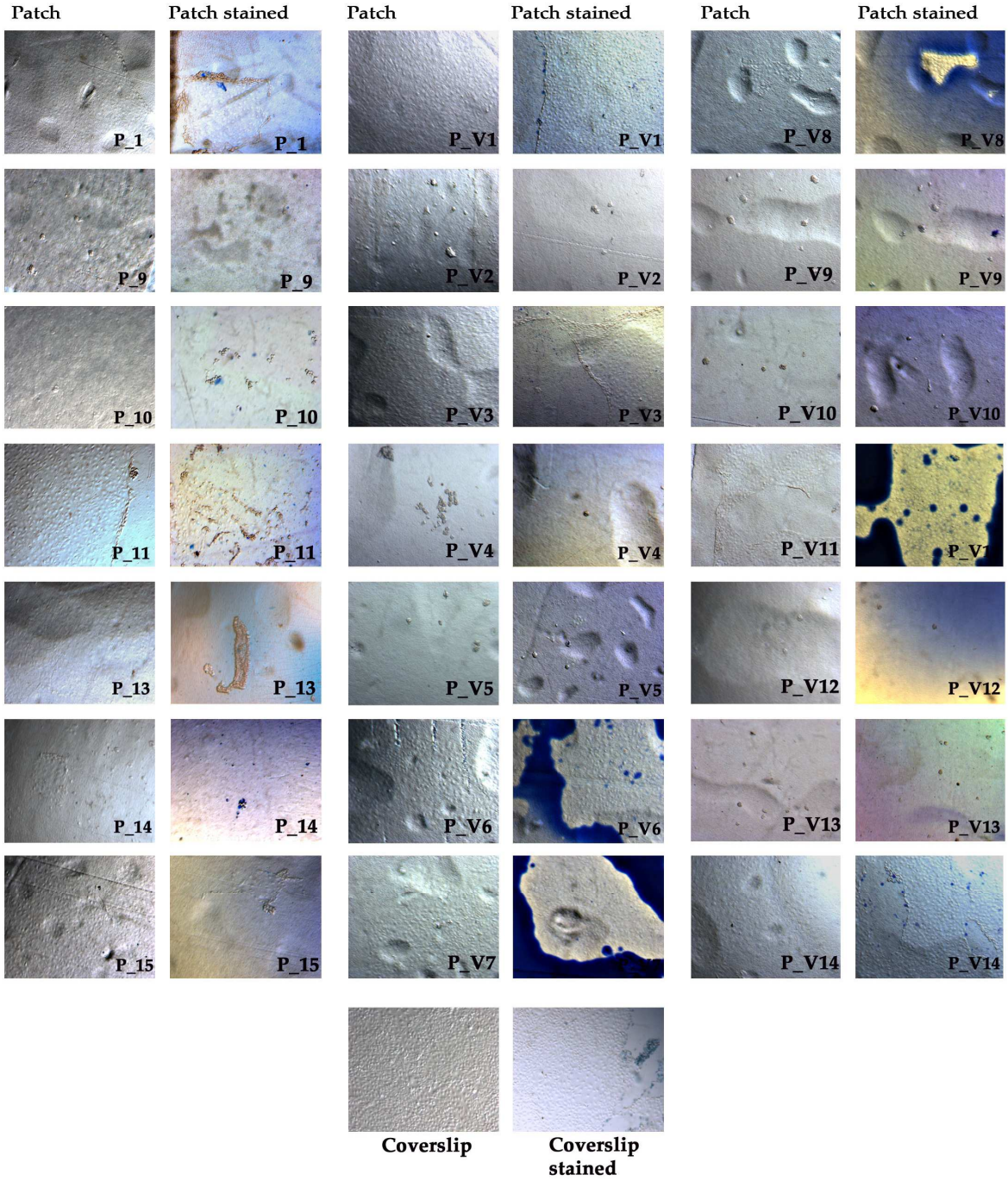

Figure 6-8. Behaviour of Ptk2 cells with respect to patch chemistry

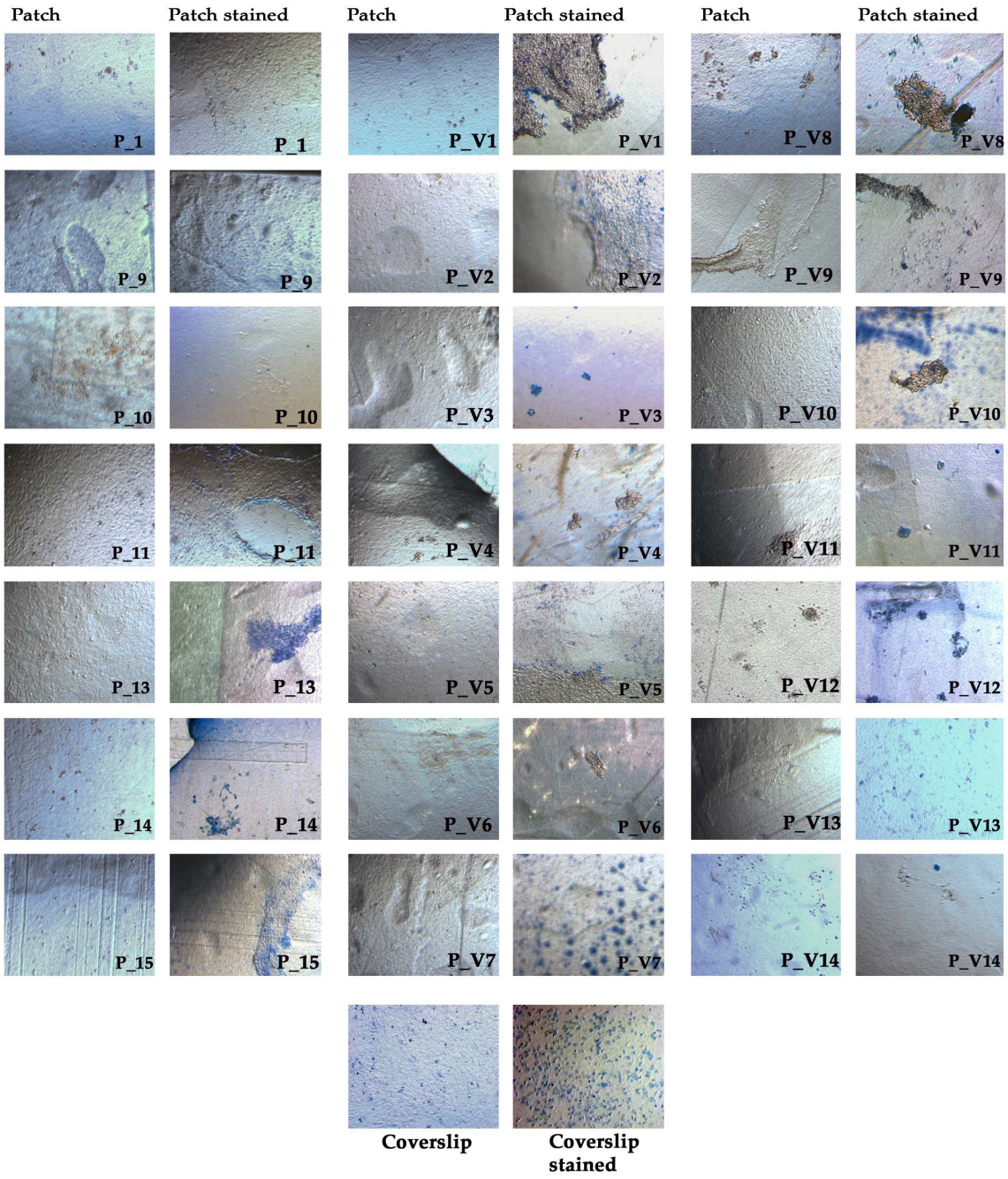

Figure 6-9. Behaviour of 3Y1 cells with respect to patch chemistry

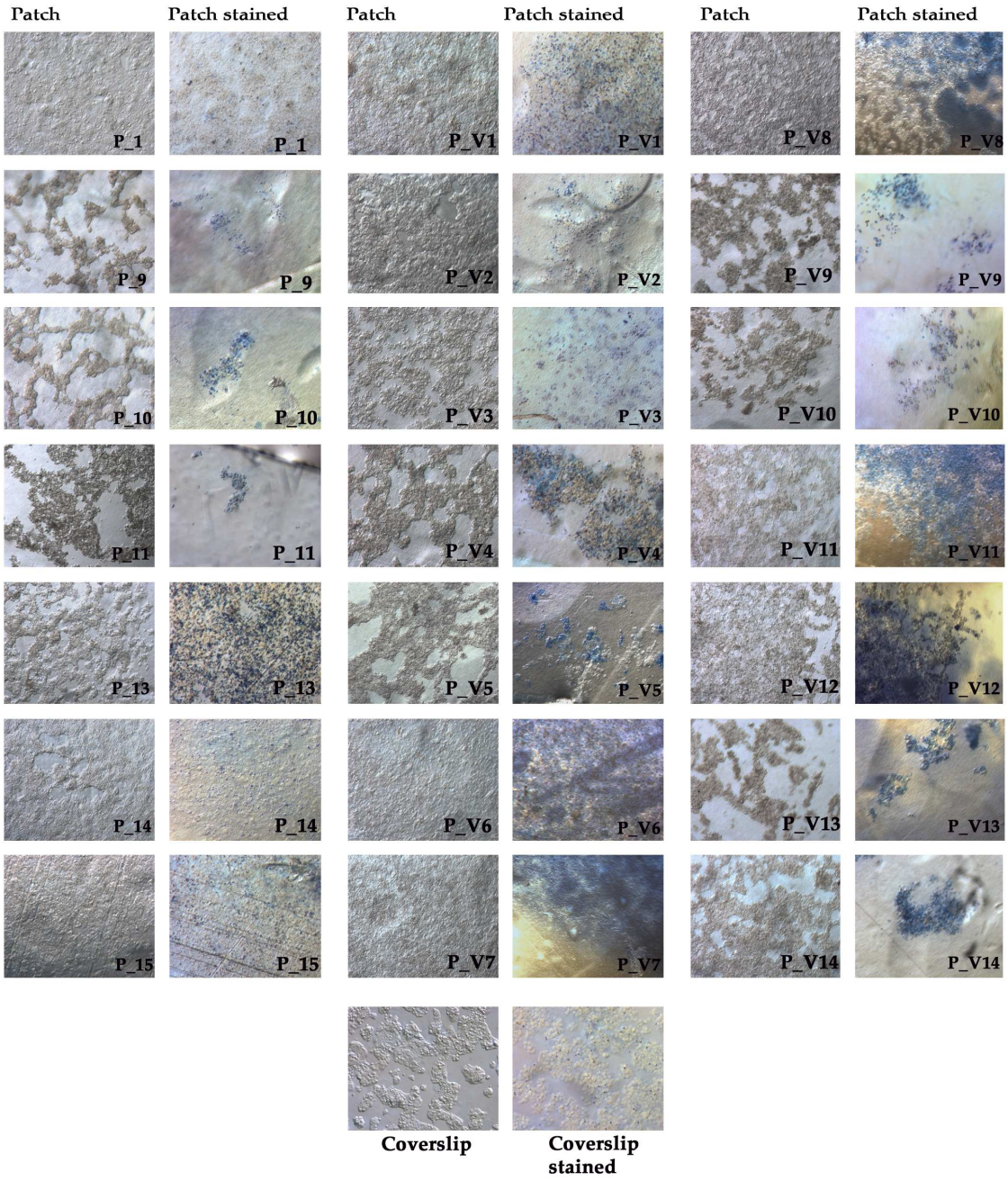

Figure 6-10. Behaviour of HepG2 cells with respect to patch chemistry

6.2 Behaviour of cells to patches with peptides synthesised on short and long spacers

Figure 6-11 to figure 6-15 compare cells grown on PP foils synthesised with highly charged peptides.

Figure 6-11 shows Hela cells, figure 6-12 shows Huh7 cells, figure 6-13 shows HepG2 cells, figure 6-14 shows PC3 cells and figure 6-15 shows PtK2 cells.

Behaviour of cells towards the surface charge of patches as well as the effect of the length of the spacers used to link these peptides to the surface of the PP foils can be observed.

(Magnification: 90X, AVAV: Non polar hydrophobic, DEDE: acidic, GQGQ: polar uncharged, KKKK: Basic, PEG: Poly ethylene glycol

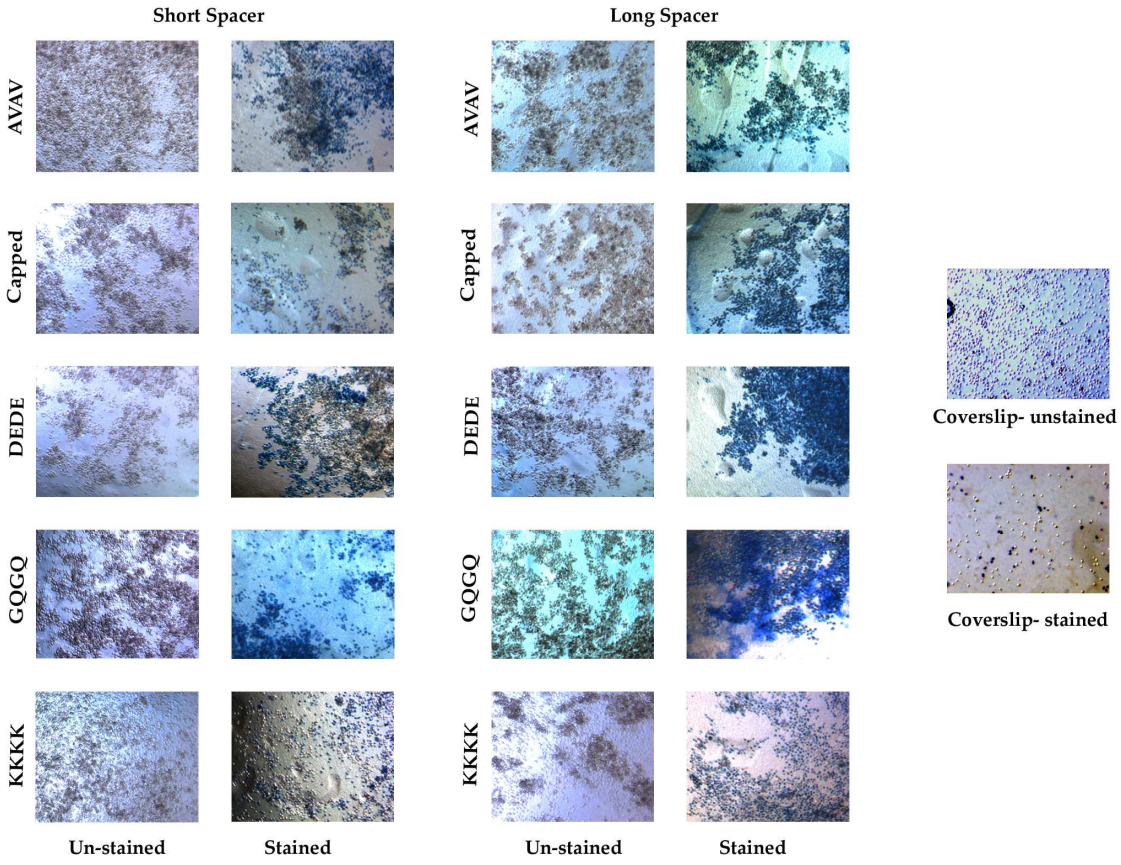

Figure 6-11. Behaviour of HeLa cells with respect to surface charge

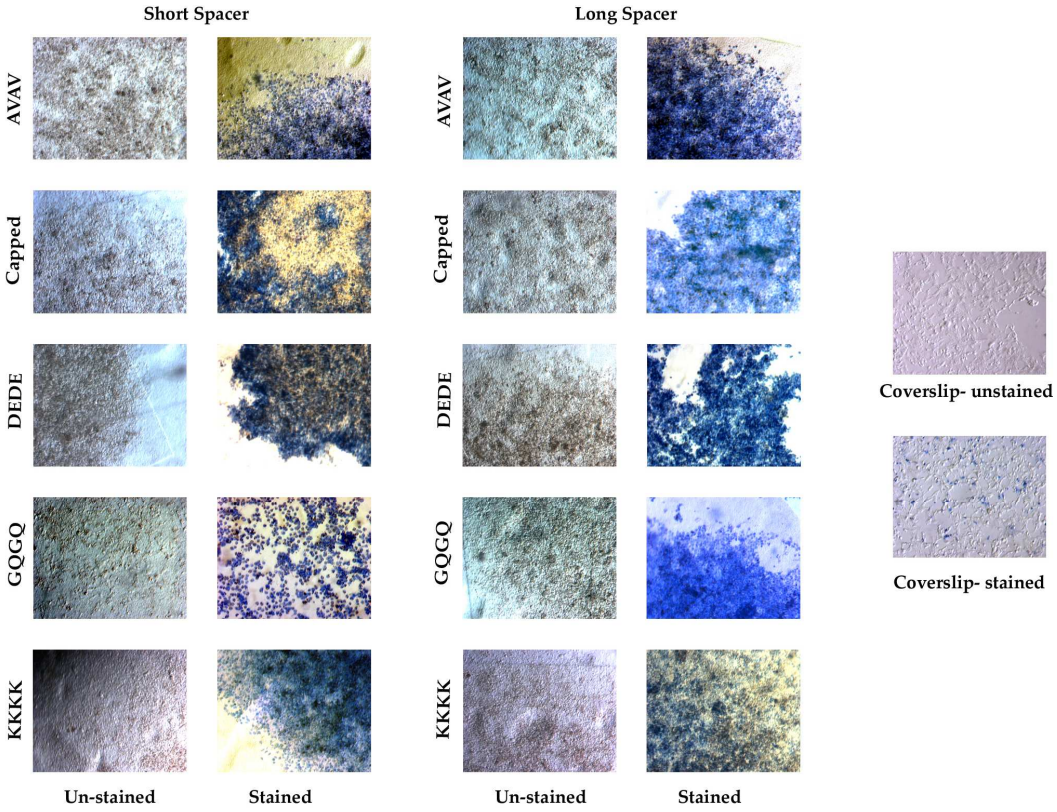

Figure 6-12. Behaviour of Huh7 cells with respect to surface charge

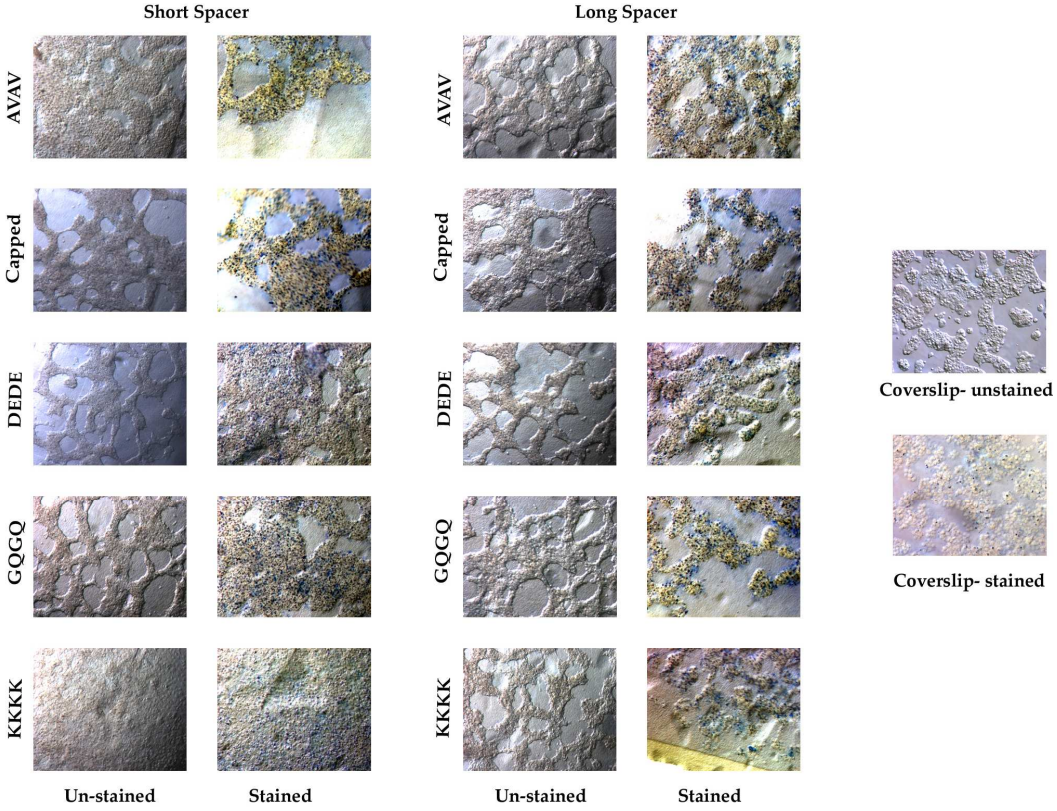

Figure 6-13. Behaviour of HepG2 cells with respect to surface charge

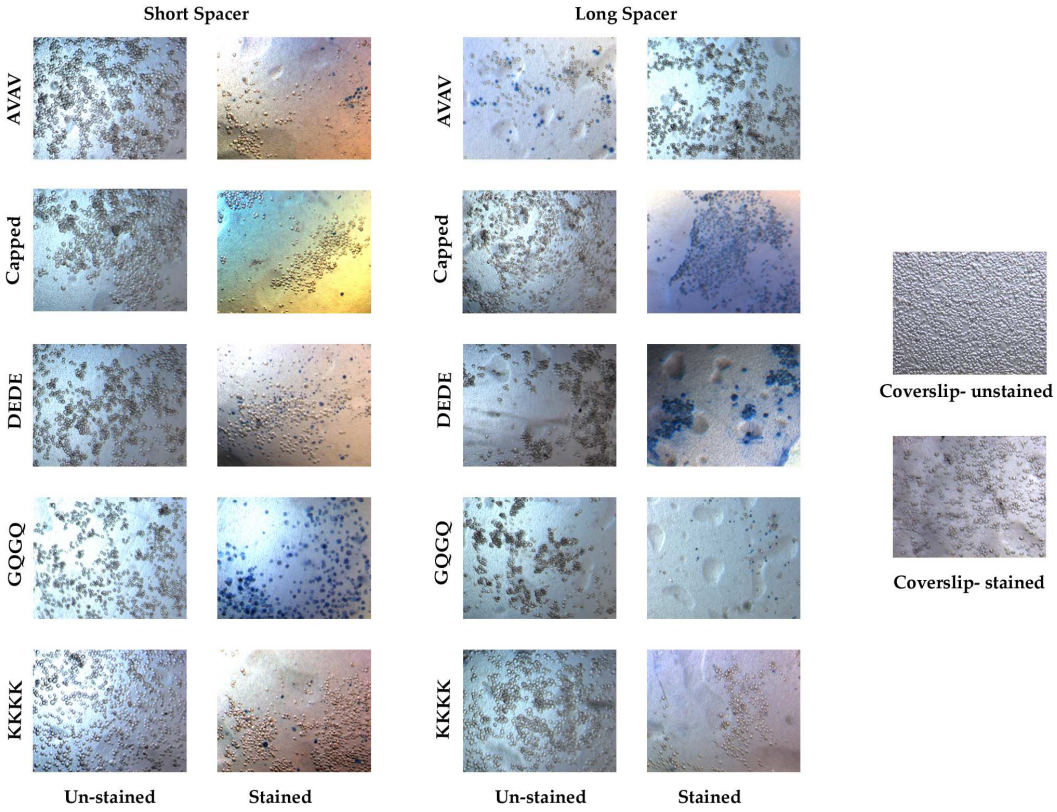

Figure 6-14. Behaviour of PC3 cells with respect to surface charge

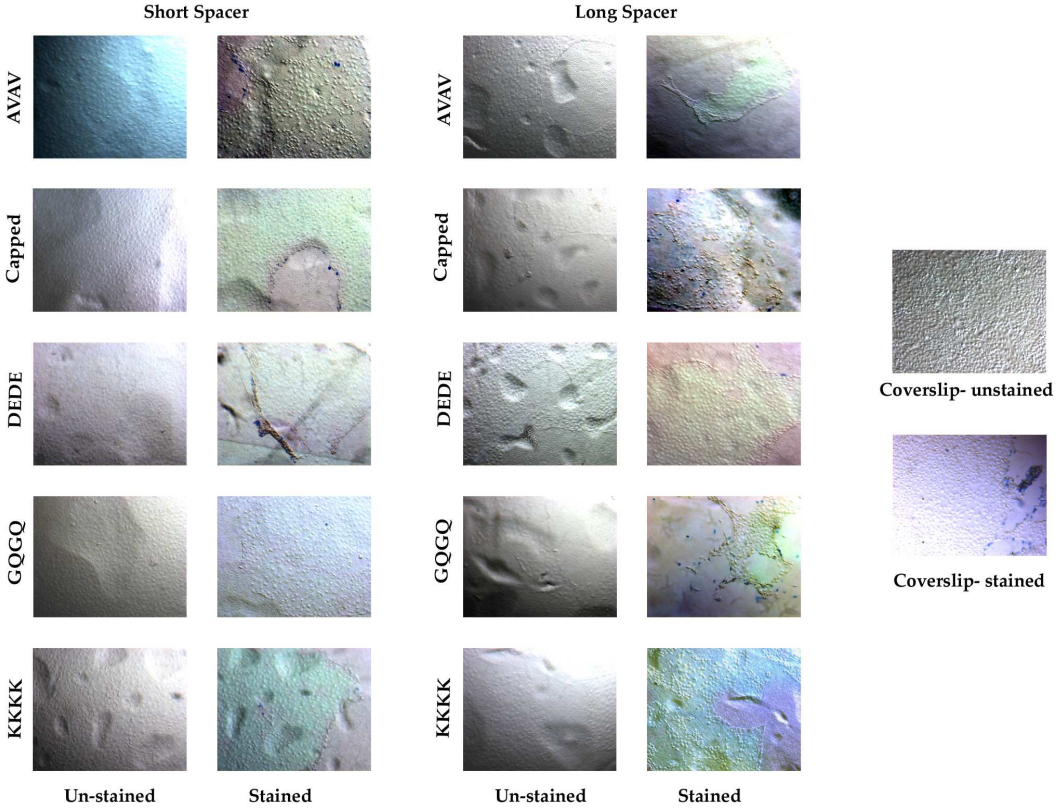

Figure 6-15. Behaviour of PtK2 cells with respect to surface charge

6.3 Comparison of cells to patches

These readings refer to Appendix 6.1.

Patch Nr			L929	Hela	SKOV 3	Huh 7	A431	A498	PC3	Ptk2	3Y1	Hep G2	Mean	Std Dev
COV	Coverslip		5	5	5	5	5	5	5	5	5	5	5.00	0.00
1	PP surface	plain foil	2	4	4	5	4	5	2	5	4	5	4.00	1.15
9	Glucosamine surface without per iodine cleavage	Aldehyde functions	2	2	2	2	2	5	2	1	2	3	2.30	1.06
10	CHO groups with Glucosamine	Aldehyde functions	2	3	4	3	3	5	1	1	1	3	2.60	1.35
11	''T'' 2h Ozone + Polymerisation	Hydroxyl functions	3	3	4	4	4	5	2	1	4	3	3.30	1.16
13	''H'' 0H- groups direct on the surface	Hydroxyl functions	3	3	4	4	1	5	3	3	3	4	3.30	1.06
14	Mat. ''T140'' 10h Ozone	Hydroxyl functions	5	4	4	4	3	5	3	2	1	4	3.50	1.27
15	Mat. ''H'' 10h Ozone	Hydroxyl functions	5	4	5	3	4	5	4	5	3	5	4.30	0.82
V1	Ozonolysis	Peroxides, Hydroxyl functions		5	4	3	4	5	4	5	4	5	4.33	0.71
V2	Polymerisation only with DMA	Methyl Groups		3	3	4	1	5	3	2	1	4	2.89	1.36
V3	Polymerisation with APMAADMA	Amino Functions		3	3	5	2	5	4	5	2	3	3.56	1.24
V4	Polymerisation with ThMAA/DMA	Hydroxyl Functions		2	2	3	2	1	3	1	4	4	2.44	1.13
V5	Polymerisation with ThMAA/DMA and CDI-activation	CDI pre activated surface		2	2	2	2	1	4	1	4	4	2.44	1.24
V6	Polymerisation with ThMAA/DMA + Diaminocyclohexane (DACH)	Amino functions		2	2	3	3	4	5	4	2	5	3.33	1.22

Patch Nr			L929	Hela	SKOV 3	Huh 7	A431	A498	PC3	Ptk2	3Y1	Hep G2	Mean	Std Dev
V7	Polymerisation with ThMAA/DMA + Jeffamin (130er)	Amino Function		4	2	2	4	5	5	4	4	5	3.89	1.17
V8	Polymerisation with ThMAA/DMA + Jeffamin (500er)	Amino Function		3	3	3	2	5	4	1	3	4	3.11	1.17
V9	Polymerisation with ThMAA/DMA + Jeffamin (130er)+ Glucosamine	Amino + hydroxyl functions		3	3	3	2	5	2	1	4	3	2.89	1.17
V10	Polymerisation with ThMAA/DMA + Jeffamin (130er) + Glucosamine + Per iodine cleavage	Aldehyde functions		4	3	2	2	4	3	1	4	2	2.78	1.09
V11	Polymerisation with ThMAA/DMA + Jeffamin (500er) + Poly-Lysine coating	Poly- Lysine		3	1	3	2	5	3	4	1	3	2.78	1.30
V12	Polymerisation with ThMAA/DMA + Jeffamin (500er) + bAlanine spots	beta Alanine		4	3	2	2	5	2	1	1	3	2.56	1.33
V13	Polymerisation with ThMAA/DMA + Jeffamin (500er) + Lysine spots	Lysine		4	3	3	2	5	2	1	2	3	2.78	1.20
V14	Ozonolysis + Boran dimethyl sulphide complex reduction	Hydroxyl functions		3	4	2	4	5	4	5	2	4	3.67	1.12
Mean			3.67	3.24	3.10	3.10	2.62	4.52	3.10	2.57	2.67	3.76		
Std Dev			1.41	0.89	1.05	1.01	1.12	1.18	1.14	1.78	1.31	0.91		

Table 6-1. Comparison of the general response of different cell lines to the surface chemistry done on the patches (for patches V1 to V 14)

Cell line	Behaviour towards PP foils (during the various steps on peptide synthesis)	Behaviour towards peptides synthesised on charged PP foils	Remarks
3Y1	poor growth	cell line not tested	
A431	poor growth	cell line not tested	
A498	excellent growth on all surface types	cell line not tested	
Hela	Fair growth	sensitive to surfaces with highly charged peptide	
HepG2	suitable for patch assay based analysis	excellent growth even on surfaces with highly charged peptides	to be used for La protein based further studies
Huh7	suitable for patch assay based analysis	sensitive to surfaces with highly charged peptide	
PC3	acceptable for patch assay based analysis	Acceptable growth	
PtK2	good growth on some patches and hardly any growth on others	excellent growth even on surfaces with highly charged peptides	
SKOV-3	acceptable for patch assay based analysis	cell line not tested	

Table 6-2. Comparison of the behaviour of the cell lines tested towards the PP foils in general and the PP foils with the highly charged peptides.

6.4 List of sequences of the peptide libraries used

Lib Mixed 65: LL XX12XX

List of 400 spots on the membrane other than the control spots

1	2	3	4	5	6	7	8	9	10
XXAAXX	XXCAXX	XXDAXX	XXEAXX	XXFAXX	XXGAXX	XXHAXX	XXIAXX	XXKAXX	XXLAXX
XXACXX	XXCCXX	XXDCXX	XXECXX	XXFCXX	XXGCXX	XXHCXX	XXICXX	XXKCXX	XXLCXX
XXADXX	XXCDXX	XXDDXX	XXEDXX	XXFDXX	XXGDXX	XXHDXX	XXIDXX	XXKDXX	XXLDXX
XXAEXX	XXCEXX	XXDEXX	XXEEXX	XXFEXX	XXGEXX	XXHEXX	XXIEXX	XXKEXX	XXLEXX
XXAFXX	XXCFXX	XXDFXX	XXEFXX	XXFFXX	XXGFXX	XXHFXX	XXIFXX	XXKFXX	XXLFXX
XXAGXX	XXCGXX	XXDGXX	XXEGXX	XXFGXX	XXGGXX	XXHGXX	XXIGXX	XXKGXX	XXLGXX
XXAHXX	XXCHXX	XXDHXX	XXEHXX	XXFHXX	XXGHXX	XXHHXX	XXIHXX	XXKHXX	XXLHXX
XXAIXX	XXCIXX	XXDIXX	XXEIXX	XXFIXX	XXGIXX	XXHIXX	XXIIXX	XXKIXX	XXLIXX
XXAKXX	XXCKXX	XXDKXX	XXEKXX	XXFKXX	XXGKXX	XXHKXX	XXIKXX	XXKKXX	XXLKXX
XXALXX	XXCLXX	XXDLXX	XXELXX	XXFLXX	XXGLXX	XXHLXX	XXILXX	XXKLXX	XXLLXX
XXAMXX	XXCMXX	XXDMXX	XXEMXX	XXFMXX	XXGMXX	XXHMXX	XXIMXX	XXKMXX	XXLMXX
XXANXX	XXCNXX	XXDNXX	XXENXX	XXFNXX	XXGNXX	XXHNXX	XXINXX	XXKNXX	XXLNXX
XXAPXX	XXCPXX	XXDPXX	XXEPXX	XXFPXX	XXGPXX	XXHPXX	XXIPXX	XXKPXX	XXLPXX
XXAQXX	XXCQXX	XXDQXX	XXEQXX	XXFQXX	XXGQXX	XXHQXX	XXIQXX	XXKQXX	XXLQXX
XXARXX	XXCRXX	XXDRXX	XXERXX	XXFRXX	XXGRXX	XXHRXX	XXIRXX	XXKRXX	XXLRXX
XXASXX	XXCSXX	XXDSXX	XXESXX	XXFSXX	XXGSXX	XXHSXX	XXISXX	XXKSXX	XXLSXX
XXATXX	XXCTXX	XXDTXX	XXETXX	XXFTXX	XXGTXX	XXHTXX	XXITXX	XXKTXX	XXLTXX
XXAVXX	XXCVXX	XXDVXX	XXEVXX	XXFVXX	XXGVXX	XXHVXX	XXIVXX	XXKVXX	XXLVXX
XXAWXX	XXCWXX	XXDWXX	XXEWXX	XXFWXX	XXGWXX	XXHWXX	XXIWXX	XXKWXX	XXLWXX
XXAYXX	XXCYXX	XXDYXX	XXEYXX	XXFYXX	XXGYXX	XXHYXX	XXIYXX	XXKYXX	XXLYXX
11	12	13	14	15	16	17	18	19	20
XXMAXX	XXNAXX	XXPAXX	XXQAXX	XXRAXX	XXSAXX	XXTAXX	XXVAXX	XXWAXX	XXYAXX
XXMCXX	XXNCXX	XXPCXX	XXQCXX	XXRCXX	XXSCXX	XXTCXX	XXVCXX	XXWCXX	XXYCXX
XXMDXX	XXNDXX	XXPDXX	XXQDXX	XXRDXX	XXSDXX	XXTDXX	XXVDXX	XXWDXX	XXYDXX
XXMEXX	XXNEXX	XXPEXX	XXQEXX	XXREXX	XXSEXX	XXTEXX	XXVEXX	XXWEXX	XXYEXX
XXMFXX	XXNFXX	XXPFXX	XXQFXX	XXRFXX	XXSFXX	XXTFXX	XXVFXX	XXWFXX	XXYFXX
XXMGXX	XXNGXX	XXPGXX	XXQGXX	XXRGXX	XXSGXX	XXTGXX	XXVGXX	XXWGXX	XXYGXX
XXMHXX	XXNHXX	XXPHXX	XXQHXX	XXRHXX	XXSHXX	XXTHXX	XXVHXX	XXWHXX	XXYHXX
XXMIXX	XXNIXX	XXPIXX	XXQIXX	XXRIXX	XXSIXX	XXTIXX	XXVIXX	XXWIXX	XXYIXX
XXMKXX	XXNKXX	XXPKXX	XXQKXX	XXRKXX	XXSKXX	XXTKXX	XXVKXX	XXWKXX	XXYKXX
XXMLXX	XXNLXX	XXPLXX	XXQLXX	XXRLXX	XXSLXX	XXTLXX	XXVLXX	XXWLXX	XXYLXX
XXMMXX	XXNMXX	XXPMXX	XXQMXX	XXRMXX	XXSMXX	XXTMXX	XXVMXX	XXWMXX	XXYMXX
XXMNXX	XXNNXX	XXPNXX	XXQNXX	XXRNXX	XXSNXX	XXTNXX	XXVNXX	XXWNXX	XXYNXX
XXMPXX	XXNPXX	XXPPXX	XXQPXX	XXRPXX	XXSPXX	XXTPXX	XXVPXX	XXWPXX	XXYPXX
XXMQXX	XXNQXX	XXPQXX	XXQQXX	XXRQXX	XXSQXX	XXTQXX	XXVQXX	XXWQXX	XXYQXX
XXMRXX	XXNRXX	XXPRXX	XXQRXX	XXRRXX	XXSRXX	XXTRXX	XXVRXX	XXWRXX	XXYRXX
XXMSXX	XXNSXX	XXPSXX	XXQSXX	XXRSXX	XXSSXX	XXTSXX	XXVSXX	XXWSXX	XXYSXX
XXMTXX	XXNTXX	XXPTXX	XXQTXX	XXRTXX	XXSTXX	XXTTXX	XXVTXX	XXWTXX	XXYTXX
XXMVXX	XXNVXX	XXPVXX	XXQVXX	XXRVXX	XXSVXX	XXTVXX	XXVVXX	XXWVXX	XXYVXX
XXMWXX	XXNWXX	XXPWXX	XXQWXX	XXRWXX	XXSWXX	XXTWXX	XXVWXX	XXWWXX	XXYWXX
XXMYXX	XXNYXX	XXPYXX	XXQYXX	XXRYXX	XXSYXX	XXTYXX	XXVYXX	XXWYXX	XXYYXX

Lib 17: LL XXX12XXX

(List of 400 spots on the membrane including the 25 control spots at the end)

1	2	3	4	5	6	7	8	9
XXXAAXXX	XXXCAXXX	XXXDAXXX	XXXEAXXX	XXXFAXXX	XXXGAXXX	XXXHAXXX	XXXIAXXX	XXXKAXXX
XXXACXXX	XXXCCXXX	XXXDCXXX	XXXECXXX	XXXFCXXX	XXXGCXXX	XXXHCXXX	XXXICXXX	XXXKCXXX
XXXADXXX	XXXCDXXX	XXXDDXXX	XXXEDXXX	XXXFDXXX	XXXGDXXX	XXXHDXXX	XXXIDXXX	XXXKDXXX
XXXAEXXX	XXXCEXXX	XXXDEXXX	XXXEEXXX	XXXFEXXX	XXXGEXXX	XXXHEXXX	XXXIEXXX	XXXKEXXX
XXXAFXXX	XXXCFXXX	XXXDFXXX	XXXEFXXX	XXXFFXXX	XXXGFXXX	XXXHFXXX	XXXIFXXX	XXXKFXXX
XXXAGXXX	XXXCGXXX	XXXDGXXX	XXXEGXXX	XXXFGXXX	XXXGGXXX	XXXHGXXX	XXXIGXXX	XXXKGXXX
XXXAHXXX	XXXCHXXX	XXXDHXXX	XXXEHXXX	XXXFHXXX	XXXGHXXX	XXXHHXXX	XXXIHXXX	XXXKHXXX
XXXAIXXX	XXXCIXXX	XXXDIXXX	XXXEIXXX	XXXFIXXX	XXXGIXXX	XXXHIXXX	XXXIIXXX	XXXKIXXX
XXXAKXXX	XXXCKXXX	XXXDKXXX	XXXEKXXX	XXXFKXXX	XXXGKXXX	XXXHKXXX	XXXIKXXX	XXXKKXXX
XXXALXXX	XXXCLXXX	XXXDLXXX	XXXELXXX	XXXFLXXX	XXXGLXXX	XXXHLXXX	XXXILXXX	XXXKLXXX
XXXAMXXX	XXXCMXXX	XXXDMXXX	XXXEMXXX	XXXFMXXX	XXXGMXXX	XXXHMXXX	XXXIMXXX	XXXKMXXX
XXXANXXX	XXXCNXXX	XXXDNXXX	XXXENXXX	XXXFNXXX	XXXGNXXX	XXXHNXXX	XXXINXXX	XXXKNXXX
XXXAPXXX	XXXCPXXX	XXXDPXXX	XXXEPXXX	XXXFPXXX	XXXGPXXX	XXXHPXXX	XXXIPXXX	XXXKPXXX
XXXAQXXX	XXXCQXXX	XXXDQXXX	XXXEQXXX	XXXFQXXX	XXXGQXXX	XXXHQXXX	XXXIQXXX	XXXKQXXX
XXXARXXX	XXXCRXXX	XXXDRXXX	XXXERXXX	XXXFRXXX	XXXGRXXX	XXXHRXXX	XXXIRXXX	XXXKRXXX
XXXASXXX	XXXCSXXX	XXXDSXXX	XXXESXXX	XXXFSXXX	XXXGSXXX	XXXHSXXX	XXXISXXX	XXXKSXXX
XXXATXXX	XXXCTXXX	XXXDTXXX	XXXETXXX	XXXFTXXX	XXXGTXXX	XXXHTXXX	XXXITXXX	XXXKTXXX
XXXAVXXX	XXXCVXXX	XXXDVXXX	XXXEVXXX	XXXFVXXX	XXXGVXXX	XXXHVXXX	XXXIVXXX	XXXKVXXX
XXXAWXXX	XXXCWXXX	XXXDWXXX	XXXEWXXX	XXXFWXXX	XXXGWXXX	XXXHWXXX	XXXIWXXX	XXXKWXXX
XXXAYXXX	XXXCYXXX	XXXDYXXX	XXXEYXXX	XXXFYXXX	XXXGYXXX	XXXHYXXX	XXXIYXXX	XXXKYXXX
10	11	12	13	14	15	16	17	18
XXXLAXXX	XXXMAXXX	XXXNAXXX	XXXPAXXX	XXXQAXXX	XXXRAXXX	XXXSAXXX	XXXTAXXX	XXXVAXXX
XXXLCXXX	XXXMCXXX	XXXNCXXX	XXXPCXXX	XXXQCXXX	XXXRCXXX	XXXSCXXX	XXXTCXXX	XXXVCXXX
XXXLDXXX	XXXMDXXX	XXXNDXXX	XXXPDXXX	XXXQDXXX	XXXRDXXX	XXXSDXXX	XXXTDXXX	XXXVDXXX
XXXLEXXX	XXXMEXXX	XXXNEXXX	XXXPEXXX	XXXQEXXX	XXXREXXX	XXXSEXXX	XXXTEXXX	XXXVEXXX
XXXLFXXX	XXXMFXXX	XXXNFXXX	XXXPFXXX	XXXQFXXX	XXXRFXXX	XXXSFXXX	XXXTFXXX	XXXVFXXX
XXXLGXXX	XXXMGXXX	XXXNGXXX	XXXPGXXX	XXXQGXXX	XXXRGXXX	XXXSGXXX	XXXTGXXX	XXXVGXXX
XXXLHXXX	XXXMHXXX	XXXNHXXX	XXXPHXXX	XXXQHXXX	XXXRHXXX	XXXSHXXX	XXXTHXXX	XXXVHXXX
XXXLIXXX	XXXMIXXX	XXXNIXXX	XXXPIXXX	XXXQIXXX	XXXRIXXX	XXXSIXXX	XXXTIXXX	XXXVIXXX
XXXLKXXX	XXXMKXXX	XXXNKXXX	XXXPKXXX	XXXQKXXX	XXXRKXXX	XXXSKXXX	XXXTKXXX	XXXVKXXX
XXXLLXXX	XXXMLXXX	XXXNLXXX	XXXPLXXX	XXXQLXXX	XXXRLXXX	XXXSLXXX	XXXTLXXX	XXXVLXXX
XXXLMXXX	XXXMMXXX	XXXNMXXX	XXXPMXXX	XXXQMXXX	XXXRMXXX	XXXSMXXX	XXXTMXXX	XXXVMXXX
XXXLNXXX	XXXMNXXX	XXXNNXXX	XXXPNXXX	XXXQNXXX	XXXRNXXX	XXXSNXXX	XXXTNXXX	XXXVNXXX
XXXLPXXX	XXXMPXXX	XXXNPXXX	XXXPPXXX	XXXQPXXX	XXXRPXXX	XXXSPXXX	XXXTPXXX	XXXVPXXX
XXXLQXXX	XXXMQXXX	XXXNQXXX	XXXPQXXX	XXXQQXXX	XXXRQXXX	XXXSQXXX	XXXTQXXX	XXXVQXXX
XXXLRXXX	XXXMRXXX	XXXNRXXX	XXXPRXXX	XXXQRXXX	XXXRRXXX	XXXSRXXX	XXXTRXXX	XXXVRXXX
XXXLSXXX	XXXMSXXX	XXXNSXXX	XXXPSXXX	XXXQSXXX	XXXRSXXX	XXXSSXXX	XXXTSXXX	XXXVSXXX
XXXLTXXX	XXXMTXXX	XXXNTXXX	XXXPTXXX	XXXQTXXX	XXXRTXXX	XXXSTXXX	XXXTTXXX	XXXVTXXX
XXXLVXXX	XXXMVXXX	XXXNVXXX	XXXPVXXX	XXXQVXXX	XXXRVXXX	XXXSVXXX	XXXTVXXX	XXXVVXXX
XXXLWXXX	XXXMWXXX	XXXNWXXX	XXXPWXXX	XXXQWXXX	XXXRWXXX	XXXSWXXX	XXXTWXXX	XXXVWXXX
XXXLYXXX	XXXMYXXX	XXXNYXXX	XXXPYXXX	XXXQYXXX	XXXRYXXX	XXXSYXXX	XXXTYXXX	XXXVYXXX
19	20		Control spots					
XXXWAXXX	XXXYAXXX		XXXAAXXX	XXXCCXXX				
XXXWCXXX	XXXYCXXX		XXXCCXXX	XXXGGXXX				
XXXWDXXX	XXXYDXXX		XXXDDXXX	XXXQQXXX				
XXXWEXXX	XXXYEXXX		XXXEEXXX	XXXRRXXX				
XXXWFXXX	XXXYFXXX		XXXFFXXX	XXXWWXXX				
XXXWGXXX	XXXYGXXX		XXXGGXXX					
XXXWHXXX	XXXYHXXX		XXXHHXXX					
XXXWIXXX	XXXYIXXX		XXXIIXXX					
XXXWKXXX	XXXYKXXX		XXXKKXXX					
XXXWLXXX	XXXYLXXX		XXXLLXXX					
XXXWMXXX	XXXYMXXX		XXXMMXXX					
XXXWNXXX	XXXYNXXX		XXXNNXXX					
XXXWPXXX	XXXYPXXX		XXXPPXXX					
XXXWQXXX	XXXYQXXX		XXXQQXXX					
XXXWRXXX	XXXYRXXX		XXXRRXXX					
XXXWSXXX	XXXYSXXX		XXXSSXXX					
XXXWTXXX	XXXYTXXX		XXXTTXXX					
XXXWVXXX	XXXYVXXX		XXXVVXXX					
XXXWWXXX	XXXYWXXX		XXXWWXXX					
XXXWYXXX	XXXYYXXX		XXXYYXXX					

Lib 20 Filter 3: PNA Lib 1 LL

1	2	3	4	5	6	7	8	9
XBaaBBX	XBaBaBX	XXaaaXX	XXcaaXX	XXgaaXX	XXtaaXX	XaXaaXX	XcXaaXX	XgXaaXX
XBacBBX	XBaBcBX	XXaacXX	XXcacXX	XXgacXX	XXtacXX	XaXacXX	XcXacXX	XgXacXX
XBagBBX	XBaBgBX	XXaagXX	XXcagXX	XXgagXX	XXtagXX	XaXagXX	XcXagXX	XgXagXX
XBatBBX	XBaBtBX	XXaatXX	XXcatXX	XXgatXX	XXtatXX	XaXatXX	XcXatXX	XgXatXX
XBcaBBX	XBcBaBX	XXacaXX	XXccaXX	XXgcaXX	XXtcaXX	XaXcaXX	XcXcaXX	XgXcaXX
XBccBBX	XBcBcBX	XXaccXX	XXcccXX	XXgccXX	XXtccXX	XaXccXX	XcXccXX	XgXccXX
XBcgBBX	XBcBgBX	XXacgXX	XXccgXX	XXgcgXX	XXtcgXX	XaXcgXX	XcXcgXX	XgXcgXX
XBctBBX	XBcBtBX	XXactXX	XXcctXX	XXgctXX	XXtctXX	XaXctXX	XcXctXX	XgXctXX
XBgaBBX	XBgBaBX	XXagaXX	XXcgaXX	XXggaXX	XXtgaXX	XaXgaXX	XcXgaXX	XgXgaXX
XBgcBBX	XBgBcBX	XXagcXX	XXcgcXX	XXggcXX	XXtgcXX	XaXgcXX	XcXgcXX	XgXgcXX
XBggBBX	XBgBgBX	XXaggXX	XXcggXX	XXgggXX	XXtggXX	XaXggXX	XcXggXX	XgXggXX
XBgtBBX	XBgBtBX	XXagtXX	XXcgtXX	XXggtXX	XXtgtXX	XaXgtXX	XcXgtXX	XgXgtXX
XBtaBBX	XBtBaBX	XXataXX	XXctaXX	XXgtaXX	XXttaXX	XaXtaXX	XcXtaXX	XgXtaXX
XBtcBBX	XBtBcBX	XXatcXX	XXctcXX	XXgtcXX	XXttcXX	XaXtcXX	XcXtcXX	XgXtcXX
XBtgBBX	XBtBgBX	XXatgXX	XXctgXX	XXgtgXX	XXttgXX	XaXtgXX	XcXtgXX	XgXtgXX
XBttBBX	XBtBtBX	XXattXX	XXcttXX	XXgttXX	XXtttXX	XaXttXX	XcXttXX	XgXttXX
10	11	12	13	14	15	16	17	18
XtXaaXX	XaXaXaX	XcXaXaX	XgXaXaX	XtXaXaX	XaaXaXX	XcaXaXX	XgaXaXX	XtaXaXX
XtXacXX	XaXaXcX	XcXaXcX	XgXaXcX	XtXaXcX	XaaXcXX	XcaXcXX	XgaXcXX	XtaXcXX
XtXagXX	XaXaXgX	XcXaXgX	XgXaXgX	XtXaXgX	XaaXgXX	XcaXgXX	XgaXgXX	XtaXgXX
XtXatXX	XaXaXtX	XcXaXtX	XgXaXtX	XtXaXtX	XaaXtXX	XcaXtXX	XgaXtXX	XtaXtXX
XtXcaXX	XaXcXaX	XcXcXaX	XgXcXaX	XtXcXaX	XacXaXX	XccXaXX	XgcXaXX	XtcXaXX
XtXccXX	XaXcXcX	XcXcXcX	XgXcXcX	XtXcXcX	XacXcXX	XccXcXX	XgcXcXX	XtcXcXX
XtXcgXX	XaXcXgX	XcXcXgX	XgXcXgX	XtXcXgX	XacXgXX	XccXgXX	XgcXgXX	XtcXgXX
XtXctXX	XaXcXtX	XcXcXtX	XgXcXtX	XtXcXtX	XacXtXX	XccXtXX	XgcXtXX	XtcXtXX
XtXgaXX	XaXgXaX	XcXgXaX	XgXgXaX	XtXgXaX	XagXaXX	XcgXaXX	XggXaXX	XtgXaXX
XtXgcXX	XaXgXcX	XcXgXcX	XgXgXcX	XtXgXcX	XagXcXX	XcgXcXX	XggXcXX	XtgXcXX
XtXggXX	XaXgXgX	XcXgXgX	XgXgXgX	XtXgXgX	XagXgXX	XcgXgXX	XggXgXX	XtgXgXX
XtXgtXX	XaXgXtX	XcXgXtX	XgXgXtX	XtXgXtX	XagXtXX	XcgXtXX	XggXtXX	XtgXtXX
XtXtaXX	XaXtXaX	XcXtXaX	XgXtXaX	XtXtXaX	XatXaXX	XctXaXX	XgtXaXX	XttXaXX
XtXtcXX	XaXtXcX	XcXtXcX	XgXtXcX	XtXtXcX	XatXcXX	XctXcXX	XgtXcXX	XttXcXX
XtXtgXX	XaXtXgX	XcXtXgX	XgXtXgX	XtXtXgX	XatXgXX	XctXgXX	XgtXgXX	XttXgXX
XtXttXX	XaXtXtX	XcXtXtX	XgXtXtX	XtXtXtX	XatXtXX	XctXtXX	XgtXtXX	XttXtXX
19	20	21	22	23	24	25	26	
XaXXaaX	XcXXaaX	XgXXaaX	XtXXaaX	XaaXXaX	XcaXXaX	XgaXXaX	XtaXXaX	
XaXXacX	XcXXacX	XgXXacX	XtXXacX	XaaXXcX	XcaXXcX	XgaXXcX	XtaXXcX	
XaXXagX	XcXXagX	XgXXagX	XtXXagX	XaaXXgX	XcaXXgX	XgaXXgX	XtaXXgX	
XaXXatX	XcXXatX	XgXXatX	XtXXatX	XaaXXtX	XcaXXtX	XgaXXtX	XtaXXtX	
XaXXcaX	XcXXcaX	XgXXcaX	XtXXcaX	XacXXaX	XccXXaX	XgcXXaX	XtcXXaX	
XaXXccX	XcXXccX	XgXXccX	XtXXccX	XacXXcX	XccXXcX	XgcXXcX	XtcXXcX	
XaXXcgX	XcXXcgX	XgXXcgX	XtXXcgX	XacXXgX	XccXXgX	XgcXXgX	XtcXXgX	
XaXXctX	XcXXctX	XgXXctX	XtXXctX	XacXXtX	XccXXtX	XgcXXtX	XtcXXtX	
XaXXgaX	XcXXgaX	XgXXgaX	XtXXgaX	XagXXaX	XcgXXaX	XggXXaX	XtgXXaX	
XaXXgcX	XcXXgcX	XgXXgcX	XtXXgcX	XagXXcX	XcgXXcX	XggXXcX	XtgXXcX	
XaXXggX	XcXXggX	XgXXggX	XtXXggX	XagXXgX	XcgXXgX	XggXXgX	XtgXXgX	
XaXXgtX	XcXXgtX	XgXXgtX	XtXXgtX	XagXXtX	XcgXXtX	XggXXtX	XtgXXtX	
XaXXtaX	XcXXtaX	XgXXtaX	XtXXtaX	XatXXaX	XctXXaX	XgtXXaX	XttXXaX	
XaXXtcX	XcXXtcX	XgXXtcX	XtXXtcX	XatXXcX	XctXXcX	XgtXXcX	XttXXcX	
XaXXtgX	XcXXtgX	XgXXtgX	XtXXtgX	XatXXgX	XctXXgX	XgtXXgX	XttXXgX	
XaXXttX	XcXXttX	XgXXttX	XtXXttX	XatXXtX	XctXXtX	XgtXXtX	XttXXtX	

Lib Varsha 1: **Repeat of RR 25 spots**

Positive Spots:

1. X X **D R R R** X X
2. X X **K R R D** X X
3. X X **K R R H** X X
4. X X **K R R K** X X
5. X X **K R R P** X X
6. X X **K R R R** X X
7. X X **K R R S** X X
8. X X **P R R C** X X
9. X X **R R R A** X X
10. X X **R R R H** X X
11. X X **R R R M** X X
12. X X **R R R N** X X
13. X X **S R R R** X X
14. X X **T R R K** X X
15. X X **V R R C** X X
16. X X **V R R K** X X
17. X X **W R R F** X X
18. X X **W R R G** X X
19. X X **W R R M** X X
20. X X **W R R R** X X

Negative Spots:

21. X X **E R R K** X X
22. X X **P R R W** X X
23. X X **R R R E** X X
24. X X **W R R A** X X
25. X X **W R R Y** X X

Lib Varsha 1: **Repeat of PNA 25 spots**

Positive Spots:

1. X t g B g B X
2. X g g B B c X
3. X g B B g t X
4. X g g B t B X
5. X c B B g g X
6. X B g t g B X
7. X B t g g B X
8. X c B g g B X
9. X g B g t B X
10. X t B g g B X
11. X t B g B g X
12. X g B B t g X
13. X g B g c B X
14. X t g B B g X
15. X g a B g B X

Negative Spots:

16. X B g t a B X
17. X B c a c B X
18. X B c c a B X
19. X B t g B B X
20. X B c t a B X
21. X B a a B B X
22. X a B a a B X
23. X c B t B t X
24. X B t t t B X
25. X B g g g B X

6.5 Sequence of pET Hu La

NCBI Human La Protein Sequence : 1619bp

```
5 prime
ggagtcgttgttgttgctgtttgtgagcctgtgcggcggcttctgtgggccggaaccttaaagatagc
cgcaatggctgaaaatggtgataatgaaaagatggctgccctggaggccaaaatctgtcatcaaattg
agtattattttggcgacttcaatttgccacgggacaagtttctaaaggaacagataaaactggatgaa
ggctgggtacctttggagataatgataaaattcaacaggttgaaccgtctaacaacagactttaatgt
aattgtggaagcattgagcaaatccaaggcagaactcatggaaatcagtgaagataaaactaaaatca
gaaggtctccaagcaaacccctacctgaagtgactgatgagtataaaaatgatgtaaaaaacagatct
gtttatattaaaggcttcccaactgatgcaactcttgatgacataaaagaatggttagaagataaagg
tcaagtactaaatattcagatgagaagaacattgcataaagcatttaagggatcaatttttgttgtgt
ttgatagcattgaatctgctaagaaatttgtagagacccctggccagaagtacaaagaaacagacctg
ctaatacttttcaaggacgattactttgccaaaaaaaatgaagaaagaaaacaaaataaagtggaagc
taaattaagagctaaacaggagcaagaagcaaaacaaaagttagaagaagatgctgaaatgaaatctc
tagaagaaaagattggatgcttgctgaaattttcgggtgatttagatgatcagacctgtagagaagat
ttacacatacttttctcaaatcatggtgaaataaaatggatagacttcgtcagaggagcaaaagaggg
gataattctatttaaagaaaaagccaaggaagcattgggtaaagccaaagatgcaaataatggtaacc
tacaattaaggaacaaagaagtgacttgggaagtactagaaggagaggtggaaaaagaagcactgaag
aaaataatagaagaccaacaagaatccctaaacaaatggaagtcaaaaggtcgtagatttaaaggaaa
aggaaagggtaataaagctgcccagcctgggtctggtaaaggaaaagtacagtttcagggcaagaaaa
cgaaatttgctagtgatgatgaacatgatgaacatgatgaaaatggtgcaactggacctgtgaaaaga
gcaagagaagaaacagacaaagaagaacctgcatccaaacaacagaaaacagaaaatggtgctggaga
ccagtagtttagtaaaccaatttttttattcattttaaataggttttaaacgacttttgtttgcggggc
ttttaaaaggaaaaccgaattaggtccacttcaatgtccacctgtgagaaaggaaaaatttttttgtt
gtttaacttgtctttttgttatgcaaatgagatttctttgaatgtattgttctgtttgtgttatttca
gatgattcaaatatcaaaaggaagattcttccattaaattgcctttgtaatatgagaatgtattagta
caaactaactaataaaatatatactatatgaaaagagcaaaaaaaaaaaaaaaaa
3 prime
```

Amino acids sequence of the Human La protein (P05455)

```
1     maengdnekm   aaleakichq   ieyyfgdfnl   prdkflkeqi   kldegwvple
      imikfnrlnr

61    lttdfnvive   alskskaelm   eisedktkir   rspskplpev   tdeykndvkn
      rsvyikgfpt

121   datlddikew   ledkgqvlni   qmrrtlhkaf   kgsifvvfds   iesakkfvet
      pgqkyketdl

181   lilfkddyfa   kkneerkqnk   veaklrakqe   qeakqkleed   aemksleeki
      gcllkfsgdl

241   ddqtcredlh   ilfsnhgeik   widfvrgake   giilfkekak   ealgkakdan
      ngnlqlrnke

301   vtwevlegev   ekealkkiie   dqqeslnkwk   skgrrfkgkg   kgnkaaqpgs
      gkgkvqfqgk

361 ktkfasddeh dehdengatg pvkrareetd keepaskqqk tengagdq
```

The amino acids in red are deleted in the Mutant La Δ2 protein.

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